SPECIFIC FORCE IN HUMAN SINGLE MUSCLE FIBRES WITH SPECIFIC REFERENCE TO AGEING

Kalakoutis, Michaeljohn

Awarding institution:
King's College London

For my family in Cyprus and in England,

My parents, Hilary and Telesforos,

And my brother, Nicholas.

‘For all the books,

On all the shelves,

It’s what the teachers are,

Themselves.’

Thank you, Roger (1938-2015)

SPECIFIC FORCE IN HUMAN SINGLE MUSCLE FIBRES WITH SPECIFIC REFERENCE TO AGEING

A thesis submitted for the degree of Doctor of Philosophy at King's College London

2017

Michaeljohn Kalakoutis

MSc, BSc (Hons)

Centre of Human and Aerospace Physiological Sciences

King's College London

Faculty of Life Sciences and Medicine

Acknowledgements

I would like to express my gratitude towards my primary supervisor, Professor Stephen Harridge, who gave me the opportunity to complete this PhD and who has supported me throughout; I have been able to develop considerably as a scientist in his lab. My secondary supervisor, Dr Julien Ochala, has always been helpful and available to answer my questions, and his productivity is something I have been able to learn from. I sincerely thank Dr Anthea Rowlerson, who oversaw my first experience working in a wet lab, during my MSc dissertation, and taught me how to carry out SDS PAGE during my PhD. Her expertise and scientific discussion have been a great source of help. I would also like to thank Dr Chibeza Agley, whose drive and dedication to his own work inspired me to pursue a doctorate of my own.

A special mention goes to the late Professor Roger Woledge, without whose guidance and academic expertise in muscle physiology, I would not have been able to complete this thesis. Roger always made difficult tasks seem easy and stressful situations seem light. Moreover, Roger made work seem like fun.

Thanks also go to Dr Tim West and Professor Nancy Curtin from the Royal College of Veterinary Medicine. Dr West taught me the technical skills I needed to get started with skinned fibre work, and Professor Curtin helped Roger develop the Matlab programme I have used to calculate the chemical compositions for the activating solutions employed in this thesis. I would like to thank Andrew Atkinson for his help conducting the ^{1}HNMR experiments. Tony Christopher provided invaluable technical support and Lindsey Marjoram assisted with obtaining muscle biopsies. I am grateful to both Tony and Lindsey for always being available to help. I would also like to thank the PhD students and academic staff at the Centre of Human and Aerospace Physiological Sciences, who have helped create a stimulating and enjoyable environment to work in. Thanks also go to those people who helped just because they could, Dr Lucas Hadjilucas and Dr Glen Lichtwark provided invaluable support developing Matlab code which I have used in this thesis. Finally, I am grateful to the people who volunteered to take part in the experiments presented in this thesis.

On a personal note, I would also like to thank my family in Cyprus and in England, whose support and love have given me the courage to reach this point.

Abstract

The loss of muscle strength in the elderly is greater than the loss of muscle mass, termed specific force (SF) loss, and indicates that a decrease in muscle quality contributes to age-related muscular weakness. The present PhD thesis has studied age-related SF loss in human skeletal muscle using a skinned single muscle fibre model.

A large variation in published skinned fibre SF measurements was found to exist in the literature. Therefore, a systematic review and meta-analysis was performed to identify factors causing this variability. The majority of publications were objectively divided into four research groups based on shared authorship. Methodological differences between research groups contributed to ~30% of the variance in the literature, suggesting that they are an important contributor to the variance in published SF values.

Different research groups use different activating solutions to study skinned fibres, and were assessed experimentally. Skinned fibres were exposed to different, but commonly used activating solutions (termed A and B). A significantly higher SF and a shorter time to half peak tension (t_{50}) was measured from the same fibres in solution B compared with solution A. The use of TES in solution B instead of Imidazole as a pH buffer largely caused the SF difference, and a lower Cl^- concentration and the use of Glutathione in solution B partly caused the faster t_{50}. These findings indicate that the use of different activating solutions likely affects the variance of published SF values.

The final study in this thesis compared SF between skinned fibres from physically active and comparatively frail elderly cohorts to a young, healthy group. MHC I fibre SF was significantly higher in solution B than A within all groups. No significant differences in SF, myosin content (SDS PAGE) or order (X-ray diffraction) were observed between groups. These findings suggest that physical activity does not affect age-related skinned fibre SF loss and that SF is related to skinned fibre myosin content.

Table of Contents

Table of Figures

List of Tables

General abbreviations

AP: Adductor Pollicis

ATP: Adenosine Triphosphate

BAP: brightness area product

CP: Creatine Phosphate

Cl^-: Chloride

CSA: cross-sectional area

D_2O: deuterium oxide

EGTA: (ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid)

EM: electron microscopy

GL: Glutathione

H^+: hydrogen ions

H_2O: water

IVMA: *In vitro* motility assay

KCL: Potassium Chloride

K-prop: Potassium Propionate

MHC: myosin heavy chain

P_0: peak isomeric force

P_i: Inorganic phosphate

pKa: negative base ten log of the acid dissociation constant

pCa: the negative log base ten, of calcium concentration

SDS-PAGE: Sodium Dodecyl Sulphate Gel Electrophoresis

SL: Sarcomere length

TES: 2-[(2-Hydroxy-1,1-bis(hydroxymethyl)ethyl)amino]ethanesulfonic acid

V_{max}: maximum shortening velocity

V_0: velocity of unloaded shortening

1 Introduction

1.1 General introduction

The proportion of elderly people over 60 years of age is increasing in modern societies worldwide. However, advances in medicine, science and technology have enabled people to live longer with disease or disability, meaning that increased life expectancy is not necessarily associated with an increased number of years of healthy living (Harper, 2014). The proportion of the budgets of healthcare organisations worldwide which is directed towards healthcare for the elderly is expected to increase with the growing elderly population. Therefore, the ageing population is considered to be a major socio-economic concern (Caley and Sidhu, 2011).

Maintaining skeletal muscle function with advancing age is associated with the maintenance of functional independence in elderly individuals (Foldvari et al., 2000). In contrast, age-related muscular weakness and frailty is associated with an increased number of falls, which comprise a large proportion of injuries in the elderly population. Falling related injuries in the elderly cost the NHS £581 million in the year 2000, equivalent to almost 20% of the total NHS expenditure on pharmaceuticals (Scuffham et al., 2003).

The functional properties of skeletal muscle are related to and influenced by morphological features such as muscle size. Indeed, a positive proportional relationship between human muscle cross-sectional area (CSA) and force has been well documented for at least the last seventy years (Haxton, 1944). Given the link between muscle size and force generation, considerable attention has been given to interventions such as exercise training, which can induce positive morphological adaptations, i.e. hypertrophy, which

have beneficial effects on force production (Trappe et al., 2000, Parente et al., 2008). Furthermore, environments such as intensive care (Puthucheary et al., 2013) or microgravity (spaceflight) (Riley et al., 2000, Widrick et al., 1999, Widrick et al., 2001) accelerate muscle adaptations which have a negative effect on force, so have been studied to better understand the onset of muscle atrophy, as well as exercise or nutritional countermeasures (Trappe et al., 2004, Trappe et al., 2008).

Much attention has been given to the age-related loss of muscle mass, termed 'sarcopenia' (Rosenberg, 1989). The onset of the reduction in muscle size and force is difficult to determine due to a lack of appropriate longitudinal studies, but it is estimated that a reduction in muscle mass occurs in middle age (~45 years old) (Janssen et al., 2000). Unfortunately, sarcopenia does not fully explain muscular weakness observed in elderly people. Studies conducted at the whole muscle level have demonstrated that the decline in muscle strength with advancing age is greater than the decline in muscle mass, indicating that an age-related decline in muscle quality occurs (Klein et al., 2001, Macaluso et al., 2002, Morse et al., 2005). A muscle's peak isometric force (P_0) normalised to its CSA yields a measurement of force produced per unit CSA, termed specific force, which is regarded as a reflection of a muscle's intrinsic contractile quality. Hence, the reduction in muscle quality, as reflected by specific force, has become a major topic in investigations into the cause of age-related muscular weakness.

The reduction in physical activity which accompanies old age is associated with multiple deleterious effects on overall health, and physical inactivity has been strongly linked with chronic disease (Mokdad et al., 2004). Analogously, studying sedentary elderly cohorts is likely to be an inappropriate model of inherent biological ageing, as the negative physiological impact of inactivity will interact with the effects of the ageing process. Therefore, physically active master athletes have been proposed as a model to

study the effects of ageing, unaffected by the negative effects of physical inactivity (Lazarus and Harridge, 2010, Pollock et al., 2015).

Due to the nature of whole muscle experiments performed *in vivo*, the mechanism of specific force loss in humans is difficult to study and cannot be studied at the level of the myofilaments. The chemically skinned fibre method has the advantage of being able to isolate and cause a single muscle fibre to contract maximally *in vitro* under highly standardised conditions, offsetting limitations of whole muscle experiments such as neural influences, the presence of non-contractile tissue and heterogeneous fibre type populations etc. The mechanism of age-related specific force loss, studied using the skinned fibre preparation, has been suggested to be a reduction in myosin content (D'Antona et al., 2003), which implies that a reduction in the number of acto-myosin cross-bridges is responsible for age-related specific force loss. Some evidence also exists suggesting that the behaviour of cross-bridges, as well as the number, affect specific force in the elderly (Lowe et al., 2001, Ochala et al., 2007).

The starting aim of the present doctoral work was to assess the impact of physical activity on the development of specific force loss in elderly individuals at the level of the myofilaments, by studying skinned skeletal muscle fibres. However, before these experiments could be performed, the lack of consistency with regard to human skinned fibre specific force values reported by different studies needed to be addressed. Thus, a systematic review and meta-analysis, in order to identify methodological factors contributing to the observed variability was undertaken. This was followed by a detailed investigation into the effects of the chemical compositions of the different activating solutions used to study skinned fibres, which identified a significant effect of the experimental solutions on the skinned fibre contractile response. Therefore, a novel approach was taken whereby the effects of two different activating solutions on skinned

fibre specific force were used as a tool to probe the potential mechanisms of age-related specific force loss with regard to cross-bridge behaviour. Experimentally, two cohorts of elderly individuals, one physically active master cyclists, another comparatively frail hip fracture patients, were compared to a control group of healthy, young individuals. The mechanisms of specific force loss with regard to skinned fibre myosin content as well as the position of the heads of the myosin molecule during contraction, obtained through preliminary data from X-ray diffraction experiments, were also assessed.

1.2 Skeletal muscle: a brief overview

1.2.1 The relation of muscle force to cross-sectional area

Skeletal muscle functions to convert chemical to mechanical energy, which occurs in the basic contractile unit of muscle, called a sarcomere. Among the protein structures which comprise a single sarcomere, the heads of myosin molecules constituting thick myosin filaments, attach to thin actin filaments to form structures termed 'cross-bridges.' During a muscle contraction myosin heads are thought to attach to and pull actin filaments towards the centre of the sarcomere, causing the sarcomere to shorten. The relative distances moved by the thick and thin filaments are greater than the length of a myosin molecule, indicating that the myosin head detaches and re-attaches to the thin filament; a process known as the cross-bridge cycle (Huxley, 1957, Huxley and Simmons, 1971). Muscle force is produced as a result of the cross-bridge cycle, which is driven by the hydrolysis of Adenosine Triphosphate (ATP) bound to the myosin molecule, hence the conversion of chemical to mechanical energy.

A myofibril is composed of repeated sarcomere structures, and the force produced by a given myofibril is the sum of the force produced by individual cross-bridges arranged

in parallel with one another. Therefore, an individual muscle fibre, itself composed of approximately two thousand myofibrils, produces force proportional to its cross-sectional area (CSA). Hence, the single fibre CSA serves as a proxy to reflect the number of cross-bridges arranged in parallel throughout the fibre. Indeed, that force measured at the whole muscle level in humans is proportional to a given muscle's anatomical CSA (ACSA) has been known for at least seventy years (Haxton, 1944). More specifically, muscle force is proportional to the physiological cross-sectional area (PCSA) which is reflective of the contractile tissue contributing to force generation, since the angle of pennation of the fibres in a given muscle is accounted for. PCSA can be affected by the density of myofilament packing, which affects the number of cross-bridges in parallel in a given ACSA. The implications of normalising force to the ACSA or PCSA are discussed in section 1.5.

1.2.2 The force-length relation and specific force

The number of cross-bridges which can be formed during a muscle contraction is dependent upon the overlap of the thick and thin filaments in a sarcomere, which in turn is determined by the sarcomere length. In their classic study, Gordon et al. (1966) determined the optimal sarcomere length for force generation in isolated frog skeletal muscle fibres. The force-length relation was shown to have characteristic phases, including an ascending phase where force increased with increasing sarcomere length until a plateau in force was reached, indicating that the optimal range of sarcomere length (2.0-2.25μm) had been found. At sarcomere lengths greater than the optimal range a decrease in force occurred until no force was produced, due to a complete absence in thick and thin filament overlap at very long (3.75μm) sarcomere lengths. In humans, an optimal sarcomere length for force development of 2.75μm has since been determined using chemically skinned, single muscle fibres (Gollapudi and Lin, 2009). Force-length is

difficult to set in humans *in vivo* due to confounding factors associated with whole muscle experiments, which are outlined in section 1.5.

Given the force-length relation in skeletal muscle, a contraction whereby the muscle length is set at an optimum sarcomere length for force production is commonly used to obtain maximal force measurements, termed an 'isometric' contraction. The word 'isometric' itself has Greek roots, being derived from the words 'iso' meaning equal and 'metro' meaning length. Due to the dependence of muscle force on the number of cross-bridges in parallel, the peak isometric force (P_0) normalised to a given muscle's CSA gives a measurement of force per unit CSA, which is regarded to be a reflection of the intrinsic contractile quality of a muscle, termed 'specific force.' The emphasis of the work presented in this thesis is on the specific force generating capacity of human skeletal muscle.

1.2.3 The force-velocity relation

The movement of muscle during a concentric contraction, i.e. the velocity of shortening, summates along sarcomeres in series with one another, so is proportional to the overall length of a muscle. Force is maximal during an isometric contraction when both ends of a muscle are fixed so that no shortening occurs. In contrast, P_0 decreases with increasing shortening velocity, exhibiting a negative non-linear relationship which was first described by Hill (1938). The heat produced by a muscle during contraction was also shown to be proportional to the shortening which occurs. The force-velocity relation for an individual fibre is calculated by causing a given fibre to perform 'isotonic' or 'isovelocity' contractions. The maximal velocity of shortening (V_{max}) is obtained via an extrapolation of the force-velocity curve, to the theoretical point where P_0 is equal to zero and shortening velocity is maximal. Alternatively, the velocity of unloaded shortening (V_0) can be calculated following a 'slack test' manoeuvre (Edman, 1979).

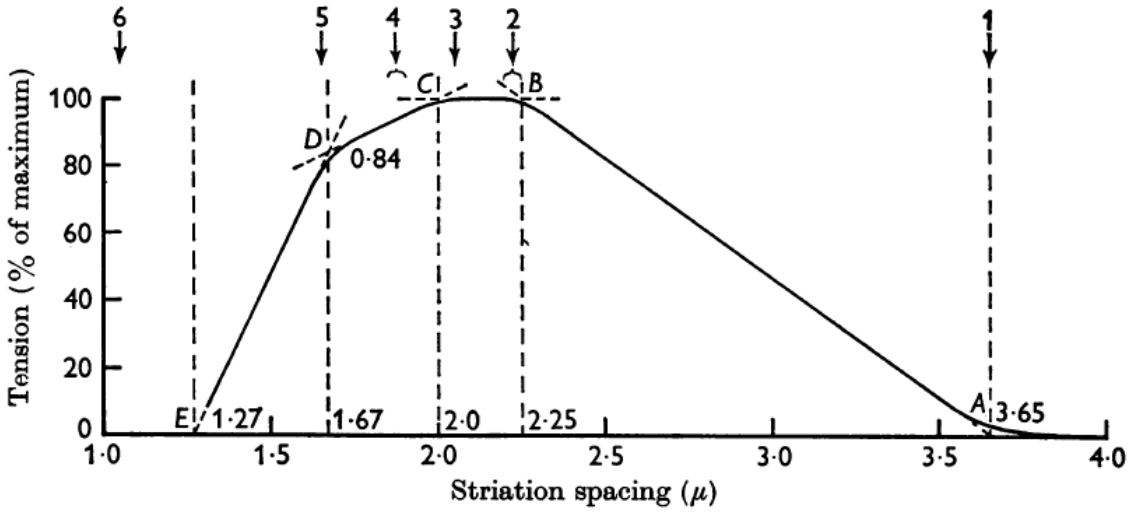


Figure 1.1. The force-length relation as demonstrated by Gordon et al. (1966) in frog muscle fibres.

The peak force was reached at an optimum sarcomere length range of 2.0-2.25um. The so called 'descending limb' can be seen to the right of the peak, where a decrease in force occurs with increasing sarcomere lengths. The ascending limb is immediately to the left of the peak, where force declines with decreasing sarcomere length.

1.2.4 Power output of concentric muscle contractions

Muscle power is the rate of work performed and is reflective of the force-velocity properties of a given muscle. Therefore, power is an indication of a muscle's functional performance. The power output of a muscle can be determined by multiplying a given P_0 with the corresponding V_{max} value, obtained from plotting a force-velocity curve.

1.2.5 Muscle fibre types

In the study of skeletal muscle, an early distinction was made between red and white muscle fibres by Ranvier (1873). Measuring the myosin ATPase activity of different muscles, Bárány (1967) observed a positive relationship between the speed of muscle shortening and the myosin ATPase activity. Indeed, the differentiation between different muscle fibres based on the pH lability of the ATPase enzyme expressed in thin (~10μm) sections of skeletal muscle revealed three distinct fibre types in humans, identified as type I, type IIA and type IIB (BROOKE and KAISER, 1970). Subsequent work revealed that the histochemical ATPase characteristics of a given muscle fibre were determined by the fibre's myosin heavy chain (MHC) isoform expression (Ennion et al., 1995, Sant'ana Pereira et al., 1995). Furthermore, the human IIB isoform was shown to not express IIB MHC, but actually expressed a human equivalent to the type IIX MHC expressed in rat skeletal muscle. Therefore, the three MHC isoforms which exist in human skeletal muscle are classified as MHC I, IIA and IIX. The fact that individual fibres can co-express multiple MHC isoforms has also been demonstrated (Ennion et al., 1995). The different fibres studied in the present thesis are referred to by their MHC isoform expression.

The mechanical properties of individual fibres have been shown to be dependent on their MHC expression. In humans, that V_{max} is faster in fibres containing MHC II than MHC I has been reported since the 1990s (Harridge et al., 1996, Stienen et al., 1996, Larsson and Moss, 1993, Bottinelli et al., 1996). Furthermore, peak power in human skeletal muscle fibres has been shown to be dependent on MHC isoform expression, increasing in the order MHC I < MHC IIA < MHC IIX (Widrick et al., 1996b). The dependence of muscle fibre power on MHC isoform expression is not surprising since V_{max}, a fundamental determinant of the force-velocity relation, is modulated by MHC isoform expression. The role of MHC isoform expression with regard to specific force is discussed later, in Chapter 2.

1.3 Age related loss of muscle mass and strength

Age related weakness has been attributed in part to a progressive loss of muscle mass, a process termed Sarcopenia (Rosenberg, 1989). Generally, elderly individuals are classified as sarcopenic if they exhibit a lean body mass measurement more than 2 standard deviations lower than that of young male or female reference groups, although several specific criterion exist (Cruz-Jentoft et al., 2010). Given the importance of muscle mass to force production, studies have tried to identify the age at which sarcopenia begins, as well as the rate of reduction in muscle mass and strength. Using cross-sections of autopsied whole Vastus Lateralis muscle, Lexell et al. (1988) reported that 10% of muscle mass in men had been lost by the age of 50, but muscle atrophy rate increased until almost 50% of muscle mass had atrophied by the age of 80. Janssen et al. (2000) observed an increasing rate of muscle atrophy at ~45 years of age (Figure 1.3), similar to Lexell et al. (1988).

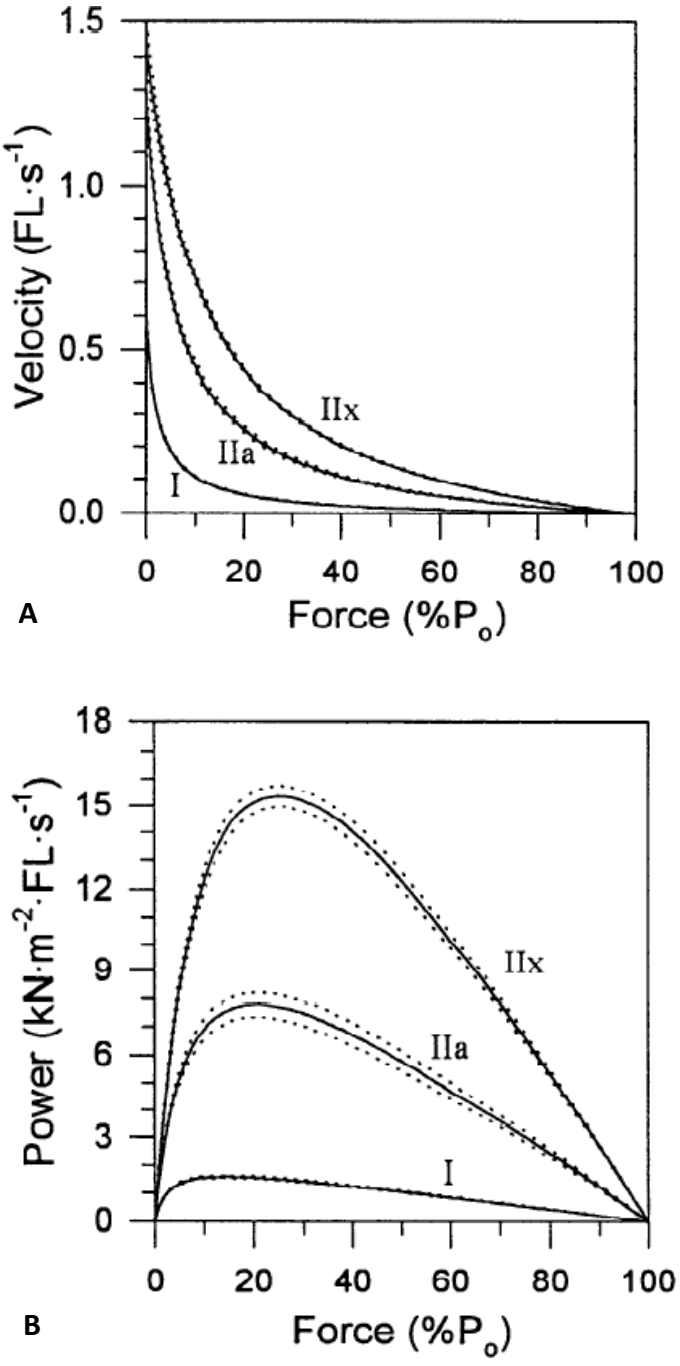


Figure 1.2. Composite force velocity and force-power curves obtained from MHC I, IIA and IIX fibres from sedentary, middle aged men (Widrick et al., 1996b).
A: The force-velocity curves were obtained using isotonic load step experiments and demonstrate the slower velocities observed in MHC I fibres compared to MHC IIA and IIX fibres. B: The force-power curve from power normalised to fibre volume. Power is calculated from the shortening velocity at a given percentage of P_0. As is illustrated, MHC I fibres exhibit lower power output than MHC IIA fibres, and MHC IIX fibres have been shown to have the highest power output in humans.

Attempts to accurately quantify the degree of muscle atrophy with age are difficult due to the cross-sectional nature of most studies. However, declines in muscle mass of ~1.2kg per decade in men aged 49-85 (Starling et al., 1999) and 1.7kg in men aged 65 and above (Baumgartner et al., 1999) have been reported by plotting the relationship between skeletal muscle mass and age. This further supports that the rate of muscle atrophy increases with age. Further complicating the interpretation of the aforementioned findings is the fact that the majority of ageing studies are performed on sedentary individuals. Therefore, the relative contributions of long-term inactivity as opposed to biological ageing, to the loss of muscle mass and strength, are difficult to determine. The issue of selection of participants for ageing research is addressed in section 1.4.

Numerous causes of sarcopenia have been documented, briefly mentioned here. Anatomically, sarcopenia is due to a reduction in the total number of muscle fibres (Lexell et al., 1983), and preferential atrophy of type II muscle fibre area has been shown to occur with age (Lexell et al., 1988). Fibre type grouping has been shown to occur in elderly individuals, and is thought to be caused by the denervation and renervation of fibres in the gradual loss of motor neurons (Lexell et al., 1986). Using spike triggered averaging from surface EMG recordings, Doherty et al. (1993) reported the age-related reduction in number of motor units was proportional to force produced during a maximal voluntary contraction, indicating muscular weakness in old age was associated with the reduction in muscle mass. As well as reductions in muscle mass and ACSA, alterations in muscle architecture via the shortening of fascicles in Gastrocnemius muscle were observed in elderly compared to young men using ultrasonography, and contributed to a reduction in PCSA (Narici et al., 2003). However, the reduction in PCSA due to changes in muscle architecture was shown not to fully account for age-related reductions in plantar flexor torque, indicating that other factors weakness (Thom et al., 2007).

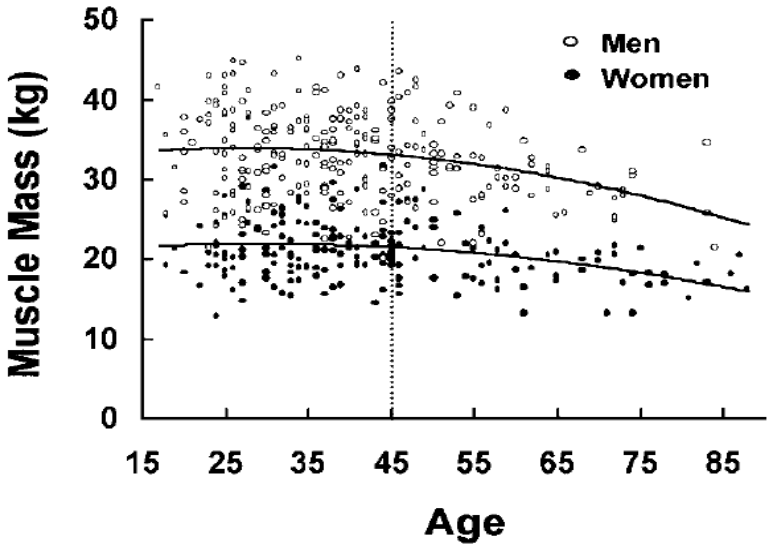


Figure 1.3. The decline in muscle mass with advancing age.
The relationship between whole body muscle mass (kg) determined using an MRI scan, and age (years). Total muscle mass remains unchanged until the age of 45, at which point the rate of muscle atrophy accelerates with age in both men and women (Janssen et al., 2000).

At the molecular level, ageing is associated with increased variability in the size of the myonuclear domain on individual muscle fibres, which may have implications for muscle protein synthesis (Cristea et al., 2010). Indeed, although basal rates of muscle protein synthesis were similar between young and elderly men, the anabolic response following ingestion of essential amino acids was lower in elderly men, suggesting a diminished anabolic response to feeding (Cuthbertson et al., 2005). Elderly men were also shown to exhibit reduced muscle protein synthesis in response to resistance exercise, as indicated by a reduced phosphorylation of anabolic signalling targets (Kumar et al., 2009), although the anabolic response was improved with increased exercise volume (Kumar et al., 2012). Further causes of sarcopenia include, but are not limited to, hormonal changes and the action of reactive oxygen species on cellular function, which are outlined in comprehensive review articles (Mitchell et al., 2015, Narici and Maffulli, 2010).

With regard to muscle function, cross-sectional (Skelton et al., 1994, Baumgartner et al., 1999) and longitudinal (Rantanen et al., 1998, Bassey, 1998) studies have reported significant annual losses of 1-2% in hand-grip strength with advancing age, which one study also reported from the quadriceps muscle (Skelton et al., 1994). The rate of strength loss has been shown, like the rate of muscle atrophy, to rapidly increase at a certain age. Vandervoort and McComas (1986) observed no significant strength changes until the age of 53, when strength rapidly decreased, while Lindle et al. (1997) reported strength losses in the 40s. Therefore, the timing of increased strength loss coincides with the onset of increased muscle atrophy (Lexell et al., 1988, Janssen et al., 2000) confirming the loss of muscle strength associated with sarcopenia.

Although strength losses with age are consistently reported in longitudinal studies (Aniansson et al., 1986, Kallman et al., 1990, Winegard et al., 1996, Bassey, 1998,

Rantanen et al., 1998) attempts to quantify this as annual strength losses are seldom performed and comparison between studies is difficult due to the different populations and muscle groups studied. These reasons also confound the comparison of the 'degree of strength loss versus rate of muscle atrophy' relationship between studies.

However, several studies compared the degree of age-related muscle atrophy they observed with the amount of strength loss. In plantarflexors and dorsiflexors (Vandervoort and McComas, 1986), the forearm (Kallman et al., 1990), knee extensors (Madsen et al., 1997, Lindle et al., 1997), and the whole body in general (compared with hand-grip strength) (Baumgartner et al., 1999), a strength decrease was observed which was only partially explained by the loss of muscle mass. One study (Greig et al., 1993) found that the decline in strength was fully accounted for by sarcopenia. However, Greig et al. (1993) performed a longitudinal study using ultrasound scans and then computed X-ray tomography in the follow-up study to determine muscle mass, introducing error which may explain their finding. Supporting that the rate of strength loss in elderly individuals is greater than the loss of muscle mass, Delmonico et al. (2009) reported reductions in isokinetic quadriceps torque two to five times greater than the loss of muscle CSA, in a large cohort of 1678 elderly individuals over a period of five years. The loss of strength relative to muscle mass in the elderly is important, since reductions in strength was an independent predictor of mobility limitation in a cohort of 3075 well-functioning elderly people (aged 70-79 years old), more so than muscle mass (Visser et al., 2005).

An important consideration to make when interpreting the aforementioned findings regarding the association between skeletal muscle mass and strength with advancing age, is that the physical activity levels of the cohorts studied were not characterised in detail. The impact of varying levels of physical activity on the relationship between muscle mass and strength is illustrated in Figure 1.4, which shows

that the loss of strength relative to muscle mass is greater in physically inactive compared to physically active individuals. The rationale for studying physically active elderly individuals is put forward in section 1.4.

The observation of a decreased ratio of muscle strength:muscle mass suggests that a reduction in muscle quality occurs at the whole muscle level in elderly individuals. Interestingly, experiments in rats have demonstrated that specific force is reduced in the Plantaris muscle of elderly (25 months old) compared to young (13 months old) rats following hypertrophy induced via an overload intervention. Furthermore, P_0 did not increase despite increased Plantaris muscle mass following overload induced hypertrophy in elderly (33 month old) rats and subsequently specific force was non-significantly reduced. These findings suggests that specific force loss occurs in elderly muscle even when atrophy does not occur (Degens et al., 1995, Degens and Alway, 2003). The reduction in muscle quality, as reflected by specific force measurements taken at the whole muscle level in humans, is discussed in section 1.5.

1.4 Biological vs Chronological Ageing

The reduction in physical activity which generally accompanies old age leads to de-conditioning, reduced fat free mass, increased body fat, and development of clinical diseases, which reduce the capacity of elderly individuals to carry out tasks of daily living, independent of the ageing process. Therefore, determining the effects of biological ageing on functional independence in sedentary humans is difficult (Tanaka and Seals, 2003). The link between chronic disease and physical inactivity, given that physical inactivity is a leading cause of death (Mokdad et al., 2004), suggests that human studies should use subjects with high physical fitness as a control group, instead of sedentary, less healthy individuals (Booth and Lees, 2006). While the importance of the relationship between

health and physical activity is accepted, the amount of exercise necessary to maintain good health is unclear (Blair et al., 2004). Indeed, a 'set point' theory has been proposed, whereby a minimum threshold of physical activity, specific to a given individual, may be required to offset the negative health impact of physical inactivity, after which point the effects of ageing as such, will be more clear (Lazarus and Harridge, 2016, Lazarus and Harridge, 2010).

Due to the practical, financial and logistical difficulties of carrying out longitudinal studies in humans, there has been a reliance on information obtained from cross-sectional studies into the effects of ageing on skeletal muscle function (Harridge and Lazarus, 2017). Due to their nature, cross-sectional studies investigating human ageing make the assumption that the elderly cohorts studied are representative of the aged physiological status of their younger counterparts. In reality, intergenerational differences are likely to mean that changes which occur with advancing age, such as physical activity patterns, are not reflected by differences observed between the young and elderly cohorts studied (Mitchell et al., 2015). Even within the young or elderly population, a continuum of differing physical activity levels exists. Furthermore, when categorised based on physical fitness, a large overlap occurred between young and elderly individuals, meaning that individuals could not be identified by their age (Lazarus and Harridge, 2010). This demonstrates the necessity to account for an individual's level of physical activity, when trying to assess the impact of biological ageing.

Master athletes have been proposed as a model of ageing, free from the deleterious, aforementioned effects of inactivity. An examination of the trend in world record athletic performances with advancing age indicates that physical performance does decline with age, even in elite elderly athletes. However, this trajectory is likely to reflect the effect of an inherent ageing process (Lazarus and Harridge, 2016). Therefore, the

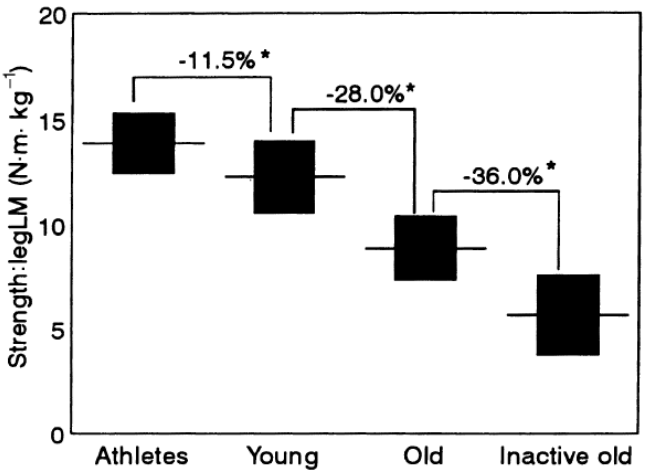


Figure 1.4. The effect of physical activity on the relationship between muscle mass and strength in young and elderly populations.
Knee extensor muscle strength expressed per kilogram of muscle mass in young female weight lifters (athletes, 26.3 years old), young non-athletic women (young, 24 years old), elderly healthy women (old, 75.5 years old) and elderly inactive patients with a previous hip fracture (inactive old, 76,8 years old) (Madsen et al., 1997). There is a significant decline in muscle strength per kilogram of muscle mass with physical status as well as age, highlighting the significance that the physical fitness of participants selected for ageing studies can have on subsequent results.

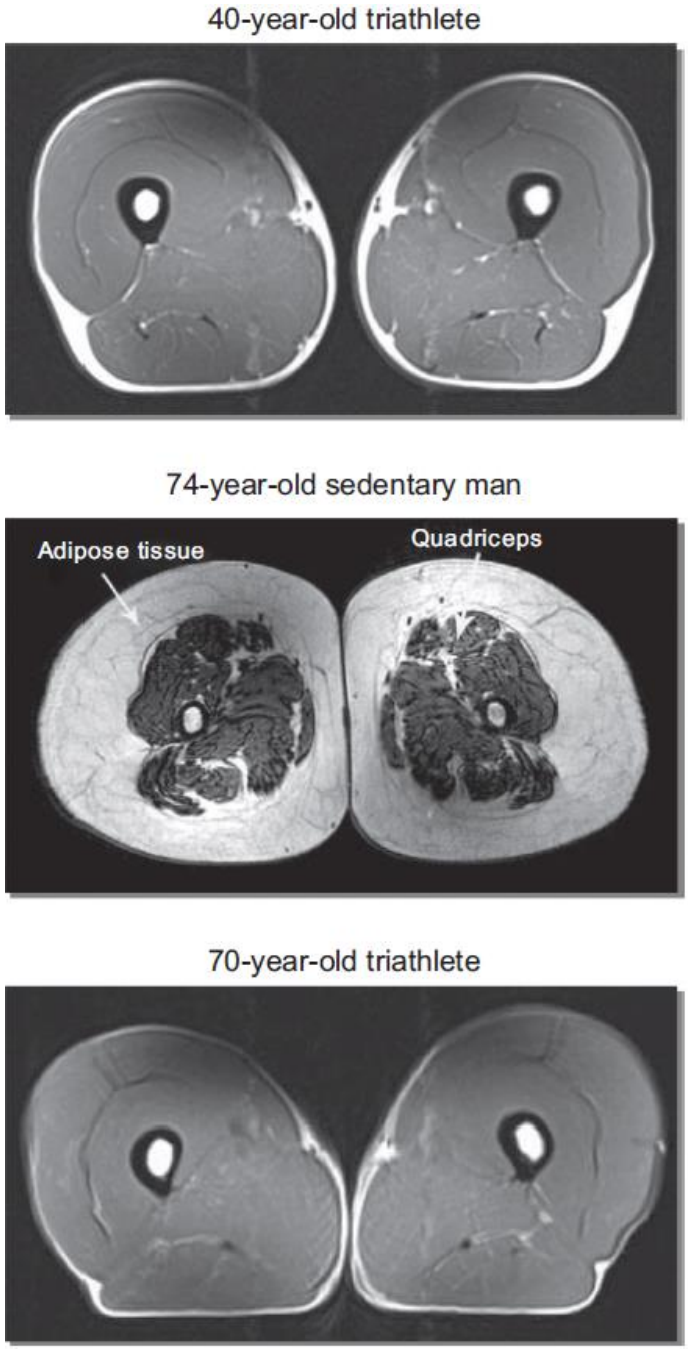


Figure 1.5. A visual representation of the effect of exercise on the preservation of muscle mass and quality.
An MRI scan illustrating the transverse section of the mid-thigh of a 40 year old triathlete (top image), compared to a 70 year old triathlete (bottom image) and a 74 year old sedentary man (middle image) (Wroblewski et al., 2011). Muscle mass was preserved in the elderly triathlete and there was a distinct lack of infiltration of fat. In contrast, the sedentary, elderly individual displayed reduced muscle mass and much greater infiltration of adipose tissue. The reduction in muscle quality in the thigh of the sedentary, elderly man which is visually represented in the above MRI scan supports that the effects of physical inactivity mask the effect of ageing as such, on skeletal muscle mass and function. In contrast, the thigh muscles of the elderly triathlete are well preserved, suggesting that master athletes offer a model of the biological ageing process free from the deleterious effects of physical inactivity.

study of physically active elderly cohorts likely represents a population in whom the effects of ageing have not been masked be health complications relating to physical inactivity Figure 1.5. Based on this information, one of the aims of the present PhD thesis was to study the development of age-related specific force loss using a group of master cyclists whose physiological function had been carefully characterised as part of previous work (Pollock et al., 2015).

1.5 Age-related specific force loss observed in whole muscle experiments

A reduction in human skeletal muscle specific force as a function of increasing age was first reported by Men et al. (1985). Equivocal results have been reported about whether there is an age-related decline in specific force at the whole muscle level in humans. The inconsistency in observing a loss of specific force with age has been attributed to methodological differences between studies (Overend et al., 1992). Indeed, a multitude of methodological factors must be taken into account to obtain a reliable specific force value at the whole muscle level, some of which are time consuming or expensive and therefore not always measured. With regard to obtaining reliable peak isometric force measurements, muscle length, co-activation of antagonist muscles and whether maximal voluntary activation occurs must be taken into account. For accurate CSA measurements, muscle fibre architecture (angles of fibre pennation) and contractile and non-contractile tissue must be accounted for, in order to obtain a reliable PCSA measurement. Hence, ranking the studies which examined the effect of age on specific force based on the validity of their methods (Table 1.1) and then assessing their results may provide a clearer picture.

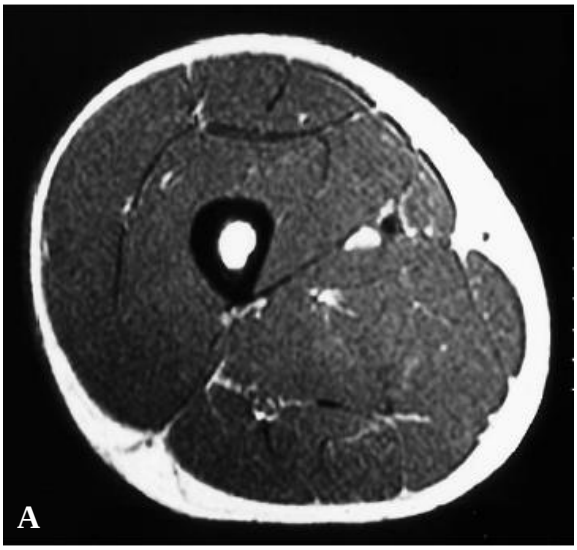
Table 1.1 shows that when methodological factors which remove confounding variables of whole muscle specific force measurements are taken into account, age-related specific force loss is observed. In particular, studies accounting for PCSA, as opposed to ACSA, observed an age-related reduction in specific force. PCSA is the total CSA of all muscle fibres in a muscle, which takes muscle volume and pennation angle of muscle fibres into account, while ACSA is the area measured from a single cross-section of muscle (Klein et al., 2001). Klein et al. (2001) showed that PCSA vs MVC correlations ($r = 0.86$ in elbow flexors and $r=0.87$ in elbow extensors) were better than anatomical cross-sectional area (ACSA) vs MVC correlations ($r = 0.83$ in elbow flexors and $r=0.80$ in elbow extensors), supporting that PCSA is a more valid measurement of contractile tissue.

However, three studies (Jubrias et al., 1997a, Phillips et al., 1992, Bruce et al., 1989b) which normalized force to ACSA, not PCSA did observe specific force loss in elderly individuals. Accurate ACSA measurements which did not largely under-represent the PCSA of a given muscle is a likely cause of these findings. Indeed, Phillips et al. (1992) and Bruce et al. (1989b) used a method to obtain adductor pollicis (AP) muscle CSA, which had been shown to correlate well ($r = 0.937$) with measurements from computed tomography scans. Skin thickness of the hand was also taken into account based on measurements from cadaveric dissection (Bruce et al., 1989a). Furthermore, the AP muscle is a parallel fibred muscle, so ACSA measurements would underestimate the PCSA less than in a multi-pennate muscle such as the quadriceps. Jubrias et al. (1997a) normalized peak isokinetic force to ACSA of the quadriceps muscle, but they used an MRI scanner which allowed them to account for non-contractile tissue.

Frontera et al. (1991) took non-contractile tissue into account but did not observe a loss of strength greater than the loss of muscle mass due to advancing age. This can be

explained by the fact that they measured muscle mass as opposed to CSA, so their measurements do not reflect specific force. Furthermore, they measured urinary creatinine excretion under the assumption that it is proportional to muscle mass, which may not be reliable in all subjects (Heymsfield et al., 1983). This indirect measure of muscle mass was used in 141 subjects, but applied the results to the full cohort of 200 subjects, indicating that significant discrepancy in muscle mass estimation is likely to have occurred.

Cannon et al. (2001) did not report specific force loss with age, however their 'elderly' cohort had a mean age of 53.9 years, which is relatively young compared to other publications. Overend et al. (1992) did not observe specific force loss from isometric contractions of elderly individuals but did in isokinetic contractions in the same study, while Frontera et al. (2000b) reported specific force loss in permeabilised fibres but not at the whole muscle level of the same study. Therefore, only one study assessed in Table 1.1 did not report age-related specific force loss having taken non-contractile tissue into account (Kent-Braun and Ng, 1999). This strongly suggests that once methodological differences are considered, the majority of the research consistently finds an effect of aging on specific force production.



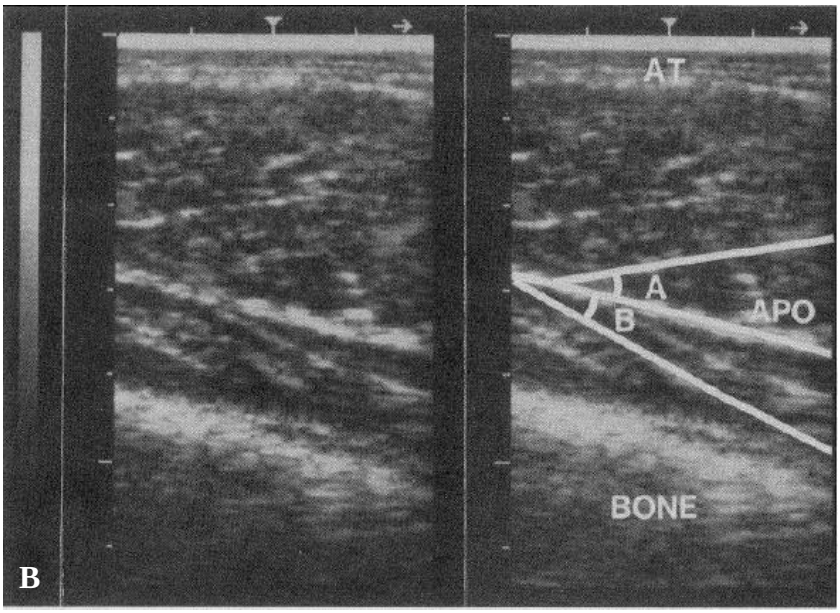


Figure 1.6. Methodological considerations required for the calculation of PCSA.
A: The MRI scan of the thigh of a young (aged 24) person (Jubrias et al., 1997b) can be used to account for non-contractile tissue, and the thigh volume can be calculated from MRI scans taken at regular intervals along the thigh. **B:** An example of an ultrasound image used to measure fascicle length and pennation angle of the triceps brachii (Kawakami et al., 1993). The image to the right is a labelled version of the image to the left. Adipose tissue (AT) and bone (Humerus) are labelled accordingly. The echoes from aponeurosis (APO) and interspaces among fascicles are indicated by the white lines. The muscle fibre pennation angles were defined as the angles between the echoes of the aponeurosis of the triceps and the echoes from interspaces among the fascicles. The pennation angles of the long and medial heads are represented by angles 'A' and 'B' respectively. PCSA is calculated as the thigh volume divided by the fascicle length.

Study	Accounted for non-muscle tissue?	PCSA	ACSA (includes non-muscle tissue)	Tendon moment arm	Agonist-antagonist co-activation	Voluntary muscle activation level	Muscle length	Pennation angle	Subject physical activity level matched	Specific force loss? ($p < 0.05$)	Muscle	Age (years) and sex of subjects
Morse et al (2005)	✓	✓	✗	✓	✓	✓	✓	✓	✗	✓	Gastrocnemius	74, Male
Klein et al (2001)	✓	✓	✗	✗	✓	✓	✓	✓	✗	✓	Elbow flexors and extensors	81, Male
Macaluso et al (2002)	✓	✓	✗	✗	✓	✗	✓	✗	✗	✓	Knee extensors and flexors	69.5, Female
Kent Braun & Ng (1999)	✓	✗	✓	✗	✗	✓	✗	✗	✓	✗	Ankle dorsiflexors	72, Male and Female
Cannon et al (2001)	✗	✗	✓	✗	✗	✓	✗	✗	✗	✗	Knee extensors	54, Male
Phillips et al (1992)	✗	✗	✓	✗	✗	✓	✗	✗	✗	✓	Adductor pollicis	80, Male and Female
Jubrias et al (1997)	✓	✗	✓	✗	✗	✗	✗	✗	✗	✓	Quadriceps	65-80, Male and Female
Lynch et al (1999)	✓	✓	✗	✗	✗	✗	✗	✗	✗	✓	Elbow and knee, flexors and extensors	65, 74, 85, Males and Females

Table 1.1 continued												
Study	**Accounted for non-muscle tissue?**	**PCSA**	**ACSA (includes non-muscle tissue)**	**Tendon moment arm**	**Agonist-antagonist co-activation**	**Voluntary muscle activation level**	**Muscle length**	**Pennation angle**	**Subject physical activity level matched**	**Specific force loss? ($p < 0.05$)**	**Muscle**	**Age (years) and sex of subjects**
Bruce et al (1989)	✗	✗	✓	✗	✗	✗	✗	✗	✗	✓	Adductor pollicis	81, Male and Female
Overend et al (1992)	✗	✗	✓	✗	✗	✗	✗	✗	✗	✗	Quadriceps, Hamstring	71, Male
Frontera et al (2000)	✗	✗	✓	✗	✗	✗	✗	✗	✗	✗	Quadriceps, Elbow extensors	77, Male
Frontera et al (1991)	✓	✗	✗	✗	✗	✗	✗	✗	✗	✗	Quadriceps, Hamstrings, Elbow extensors	69, Males and Females

Table 1.1. The observation of age-related specific force loss at the whole muscle level, in relation to the methodological measurements accounted for when measuring specific force.

A given publication is listed in the far left column, and the methodological factors listed in subsequent columns to the right. A green tick or red cross indicate that a given measurement was or was not taken respectively. Generally, specific force loss was observed in studies with more valid PCSA measurements, suggesting that differences between studies can be explained by methodological differences.

1.6 The permeabilised skeletal muscle fibre technique

To overcome the limitations of *in vivo* approaches, researchers have used *in vitro* methods to study human muscle function and specific force. This has been achieved by investigating the properties of segments of single fibres, usually obtained from a muscle biopsy sample. Pioneered during the 1970s, chemical skinning dissolves the lipid bilayer of a single muscle fibre by means of detergents such as Triton X-100, but leaves the myofilament lattice functionally intact (Stienen, 2000).

The confounding variables of whole muscle experiments, such as heterogeneous fibre type populations, the presence of non-contractile tissue, neural influences and differences in muscle fibre architecture, are offset using the chemically skinned single fibre model. The CSA and P_0 of an individual fibre can be measured and the MHC expression determined, allowing for a detailed characterisation of an individual muscle fibre's intrinsic force generating capacity. Once skinned, the myofilaments are exposed to experimental solutions, described in detail in Chapter 4, which control a given fibre's intracellular environment and allow for direct investigation of the activity of the myofibrillar proteins during contraction (Larsson et al., 1997, Frontera et al., 2000a).

The chemically skinned fibre technique is used to model the contractile function of the myofilaments for a single muscle fibre *in vivo*. For practical reasons, intact skeletal muscle fibre contractile measurements cannot currently be taken in humans. Therefore, the comparison of skinned fibre specific force in humans is limited to a direct comparison with whole muscle specific force. As discussed in section 1.5, a multitude of methodological factors must be taken into account in order to accurately measure whole muscle specific force. O'Brien et al. (2010) measured specific force from the human quadriceps muscle with great accuracy and report that specific force was similar across

adult men and women and pre-pubertal boys and girls, being approximately 560 kPa in each group. In contrast, the highest mean human, skinned fibre specific force value reported from young healthy individuals is 331 kPa and 367 kPa for type I and IIA fibres respectively, measured at 12°C (Yu et al., 2007). The discrepancy in skinned fibre and *in vivo* skeletal muscle specific force is at least in part due to the sub-physiological temperatures used to study skinned fibres, which keep the fibres from breaking, but lower the force produced. Methodological factors affecting skinned fibre force measurements are discussed further in Chapter 2.

Currently, the best way of comparing skinned fibre contractile properties with those observed *in vivo* is by comparing skinned and intact fibres from animal models (Curtin et al., 2015). However, as will be discussed extensively in Chapter 2, specific force values published by different research groups vary greatly, suggesting that the methodological practice of a given research group influences the skinned fibre contractile response. Therefore, skinned and intact fibre contractile data from the same research group and preferably the same animal would be required to provide a direct comparison of data obtained using the two techniques.

In this regard, Curtin et al. (2015) measured skinned fibre specific force and power from intact and skinned rabbit skeletal muscle tested at the same experimental temperature (25°C), since force and power generation are sensitive to temperature (Rall and Woledge, 1990, Woledge et al., 2009). Skinned and intact fibre specific forces were similar across a range of CSA values, indicating that the results were not influenced by the size of the fibre selected for experimentation. Skinned and intact fibre power normalised to volume was also similar between the two preparations. Furthermore, since P_0 is proportional to the contractile tissue of a muscle fibre, the authors normalised power to P_0 to eliminate any potential errors in CSA or volume calculations, and observed that

the ‘relative power’ was also similar between the two preparations. These findings support that the skinned fibre technique can be used to study *in vivo* muscle function.

1.7 Contractile measurements reported from human, chemically skinned skeletal muscle fibres

The methodological factors which must be taken into account when measuring *in vivo* specific force from whole muscle have been discussed in section0. Confounding variables of whole muscle experiments such as heterogeneous fibre type populations, presence of connective tissue, differences in muscle fibre architecture and neural influences, are offset using chemically skinned single muscle fibres. The skinned fibre technique allows for direct investigation of the activity of myofibrillar proteins during a muscle contraction (Larsson et al., 1997, Frontera et al., 2000a).

Like the equivocal results in whole muscle experiments, mixed results regarding the occurrence of age related specific force loss have been reported from studies using chemically skinned single muscle fibres. Given that physical activity is an important determinant of the contractile properties of skeletal muscle, the following literature review groups discussion points based on the general physical activity profiles of the elderly participants studied. Hence, the impact of studying sedentary, physically active or mobility-limited elderly cohorts is addressed. Where relevant, results assessing the impact of physical activity interventions on skinned fibre specific force from young individuals is considered. Furthermore, the mechanism underpinning the observed trends in reported specific force between young and elderly individuals is discussed with regard to skinned fibre myofibrillar protein content.

1.7.1 The effect of non-weight bearing on skinned fibre specific force

Interventions which prevent weight bearing on a given limb have been used as a model of accelerated ageing, as they enable the study of the acute or chronic effects of disuse on the contractile properties of skeletal muscle. Following two weeks of unilateral lower limb cast immobilisation, healthy young (n = 9; mean age: 24) and elderly (n = 8; mean age: 67) men displayed reductions in Vastus Lateralis MHC I fibre CSA and a significant decrease in MHC IIA fibre CSA. P_0 and specific force significantly decreased in MHC I fibres and particularly MHC IIA fibres in both groups to a similar degree, with no significant differences in specific force observed between the two groups (Hvid et al., 2011). Furthermore, young (n = 11; mean age: 24) and elderly (n = 11; mean age: 67) men who underwent four days of unilateral lower limb disuse in a knee brace exhibited significant reductions in MHC I fibre specific force, while MHC IIA fibre specific force was only lower in young men (Hvid et al., 2013). Importantly, the similar effects of non-weight bearing on the specific force in skinned fibres from young and elderly individuals suggests, that interventions carried out in young individuals can mimic the effects of muscle disuse in the elderly.

However, the validity of the aforementioned results is difficult to determine, since sarcomere length and experimental temperature were not formally measured and skinned fibre force has been shown to be sensitive to both parameters (Gollapudi and Lin, 2009) (Stienen et al., 1996). Widrick et al. (2002) reported that MHC I Soleus fibre CSA, P_0 and specific force were significantly decreased in young women (n = 4; age = 26) who underwent twelve days of unilateral lower limb suspension. This indicates that short-term non-weight bearing can induce specific force loss, although type IIA fibres were unaffected.

Exposing participants to a 6° head down tilt, bed rest model, hereby referred to simply as 'bed rest,' has been used to induce non-weight bearing for durations greater than two weeks. A seventeen day period of bed rest in middle aged (n = 8; mean age: 43) men did not affect Soleus MHC I fibre specific force, since P_0 decreased in proportion to fibre CSA, (Widrick et al., 1997). Consistent with their bed rest study, Widrick et al. (1999) reported that Soleus MHC I and IIA fibre specific force was unaffected in male astronauts (n = 4; mean age: 43), despite significant decreases in fibre diameter and P_0 in both fibre types following seventeen days of spaceflight. No changes were observed in Gastrocnemius fibres (Widrick et al., 2001). Thus, reductions in the morphological features of single fibres have been observed due to periods of non-weight bearing less than three-weeks, which are associated with a reduced absolute force generating capacity, but not reduced intrinsic contractile quality. Potential reasons for the lack of observation of reduced skinned fibre specific force from the studies in microgravity is that spaceflight is a model of non-weight bearing, but not physical inactivity, as the astronauts' physical activity levels were not controlled for.

In a bed rest study of long duration, thirty-seven days of bed rest decreased MHC I and IIA Vastus Lateralis fibre specific force by 44.6% and 41.5% respectively, in young men (n = 3; mean age: 28) (Larsson et al., 1996). The impact of a long duration of eighty-four days of bed rest was studied in young men (n = 6; mean age: 32), and caused a significant reduction in Vastus Lateralis MHC I and IIA fibre diameter and P_0. MHC I fibre specific force was significantly reduced by 28%, while the same trend was not significant in MHC IIA fibres (Trappe et al., 2004). Consistent with these findings, young women (n = 7; mean age: 34) who underwent sixty days of bed rest exhibited significantly reduced diameter and P_0 in Vastus Lateralis MHC I and IIA fibres, and Soleus MHC I fibres. Specific force was significantly reduced by 16% and 15% in MHC I Vastus

Lateralis and Soleus muscle fibres respectively (Trappe et al., 2007) (Trappe et al., 2008). Collectively these findings indicate that specific force loss occurs following a period of non-weight bearing greater than one month, primarily in MHC I fibres.

In contrast to the effect of exposure to bed rest alone, the incorporation of a resistance exercise countermeasure in young men (n = 6; mean age: 32) who underwent eighty-four days of bed rest prevented decreases in Vastus Lateralis MHC I or IIA fibre diameter. Indeed, fibre diameter increased non-significantly in both fibre types. No significant differences in P_0 or specific force were observed, although these measurements were lower in MHC I fibres compared with before bed rest (Trappe et al., 2004). Young women who underwent sixty days of bed rest with a concurrent resistance and aerobic exercise countermeasure did not exhibit changes in MHC I or IIA Vastus Lateralis fibre diameter, P_0 or specific force (Trappe et al., 2007). Soleus MHC I fibres exhibited reduced diameter and P_0 but not specific force (Trappe et al., 2008). These findings support that physical activity prevents the negative effects of non-weight bearing on the contractile properties of skinned skeletal muscle fibres.

If long-term non-weight bearing studies accurately reflect the effects of accelerated ageing on skeletal muscle, the aforementioned results suggest that specific force loss occurs at the single fibre level due to prolonged periods of muscle disuse. However, these negative effects were ameliorated and muscle function was maintained as a result of exercise. In contrast, while negative changes in skinned fibre morphological features and absolute force occurred, specific force loss following shorter term non-weight bearing was not consistently observed. This potentially indicates that a relatively extreme non-weight bearing stimulus is needed to induce changes to the intrinsic contractile strength of a muscle fibres, as opposed to only morphological changes. Furthermore, the fact that young people have been shown to exhibit specific force loss

following immobilisation or bed rest suggests that the mechanism of specific force loss could be studied in young people.

1.7.2 The effect of ageing on skinned fibre specific force: findings from sedentary, elderly cohorts

Studies investigating the effects of ageing on skinned fibre contraction typically do not contain a detailed record of the physical activity profile of the young and elderly cohorts studied. Therefore, the following sections discuss findings from studies which have been grouped based on the general physical activity profiles (i.e. sedentary, master athlete etc.) of the cohort studied.

In sedentary, elderly cohorts, several studies have observed specific force loss in both MHC I and IIA fibres. Larsson et al. (1997) reported significantly lower specific force in MHC IIA but not MHC I, chemically skinned Vastus Lateralis fibres from sedentary elderly (n = 2; age range: 73-81) compared to young (n = 2; age range: 25-31) men. Please note, the results from freeze dried fibres are not considered here, since the freeze drying process was shown to impair skinned fibre force generation (Larsson et al., 1997). Frontera et al. (2000a) studied a higher number of subjects and observed that compared to young men (n = 7; mean age: 37), specific force was significantly lower in both MHC I and IIA Vastus Lateralis fibres from elderly men (n = 12; mean age: 74). A later study also showed that specific force was significantly lower in skinned MHC I and IIA Vastus Lateralis fibres from elderly men (n = 6; mean age: 66) compared to young men (n = 6; mean age: 32) (Ochala et al., 2007).

Some evidence exists supporting that the onset of specific force at the single fibre level may be influenced by gender. No difference in MHC I Vastus Lateralis fibre specific force was found between sedentary young women (n = 7; mean age: 27) and two groups of elderly women (both groups, n = 7; mean age: 74) (Frontera et al., 2003), in contrast

to observations made in men by the same group (Frontera et al., 2000a). Suggesting that elderly women may be less susceptible to specific force loss, MHC I Vastus Lateralis fibre specific force was found to be higher in elderly women (n = 12; age range: 65-85) than men (n = 10; age range: 65-85) (Yu et al., 2007), although specific force was still significantly lower in elderly compared with young men (n = 7; age range: 20-43) and women (n = 7; mean age: 72). Furthermore, both MHC I and IIA fibre specific force had previously been found to be significantly lower in elderly women compared with elderly men (n = 12; mean age: 74) (Frontera et al., 2000a). Therefore, these findings do not clearly indicate whether the decline in specific force differs between elderly women compared to men, or whether specific force is higher in elderly women compared to men.

In contrast to the aforementioned findings, several studies have reported no significant differences between young and elderly individuals in skinned fibre specific force. Trappe et al. (2003) studied life-long sedentary individuals, removing the confounding variable of differences in physical activity between the young and elderly groups. No significant differences in MHC I or IIA Vastus Lateralis fibre specific force were observed, except that MHC IIA specific force was significantly lower in young men (n = 6; mean age: 25), compared to young women (n = 6; mean age: 25) and elderly men (n = 6; mean age: 80) and women (n = 6; mean age: 78). Consistent with these findings, no differences were observed in Vastus Lateralis MHC I and IIA specific force between non-exercising young (n = 9; mean age: 21) and elderly women (n = 6; mean age: 85) (Raue et al., 2009), or healthy young (n = 9; mean age: 24) and elderly (n = 8; mean age: 67) men (Hvid et al., 2011).

Overall, clear disparity exists in the literature regarding whether or not specific force loss occurs in human MHC I and IIA skinned skeletal muscle fibres from the elderly vs young sedentary or non-exercising population. These equivocal results contradict the

consistent observation that specific force loss occurs in young individuals following non-weight bearing interventions of long duration. Therefore, the models of accelerated ageing in skeletal muscle in young participants may not accurately reflect the effects of a sedentary lifestyle. Another possibility is that the level of physical activity required to maintain healthy muscle function, or at least prevent the deleterious effects of an inactive lifestyle, differs between different individuals (Lazarus and Harridge, 2016). This would be expected to result in the onset of specific force loss at the myofilament level in some but not all elderly individuals. The equivocal results reported by different authors have also been attributed to the varying physical activity levels of the individuals studied. Indeed, neuromuscular activity has been shown to affect the contractile properties of human skinned fibres in young (Meijer et al., 2015) and elderly subjects (D'Antona et al., 2007), the physical activity level of the individuals comprising an experimental group is likely to affect the specific force results.

1.7.3 The effect of physical activity on skinned fibre specific force in elderly cohorts

D'Antona et al. (2007) studied the impact of varying levels of physical activity on the development of specific force with advancing age. Vastus Lateralis fibres were studied from young subjects (n = 5; mean age: 30) who were recreationally active and compared with fibres obtained from three elderly groups (mean age: 73) with distinct physical activity patterns: 1) sedentary but functionally independent elderly subjects (n = 3); 2) elderly subjects who walked at least two hours per day (n = 4); 3) elderly endurance trained subjects (n = 3). Specific force was not significantly different in the elderly endurance trained group compared to the young controls. In contrast, MHC I fibre specific force in the elderly control group was significantly weaker than in young controls, and both MHC I and IIA specific force was significantly lower in the sedentary elderly group

compared to those in young controls. Furthermore, specific force measurements from MHC I and IIA fibres of elderly immobilised subjects (n = 2; aged 70 and 72) from their previous work (D'Antona et al, 2003) were included for comparison and the specific force loss observed was greater still (Figure 1.7). These data suggest a clear trend exists between the level of daily physical activity and the regulation of specific force in old age. Consistent with these findings, specific force from Vastus Lateralis MHC I and IIA fibres was not significantly different between young (n = 8; age range: 18-33) and elderly (n = 9; 53-77) individuals who had a long-term sprint training background and who were still sprint-training at the time of study (Korhonen et al., 2006).

Interestingly, the higher specific force observed in endurance trained compared to sedentary elderly individuals is not reflected in results observing no difference in skinned fibre specific force between endurance trained and untrained young (Harber and Trappe, 2008, Fitts et al., 1989) and middle aged (Widrick et al., 1996a) men. However, specific force measured from skinned MHC I and IIA Gastrocnemius fibres from young, recreationally active individuals and trained runners, increased due to endurance running training (Harber et al., 2004, Trappe et al., 2006). This suggests that endurance training does improve specific force, supporting the findings that skinned fibre specific force is maintained in endurance trained elderly individuals (D'Antona et al., 2007).

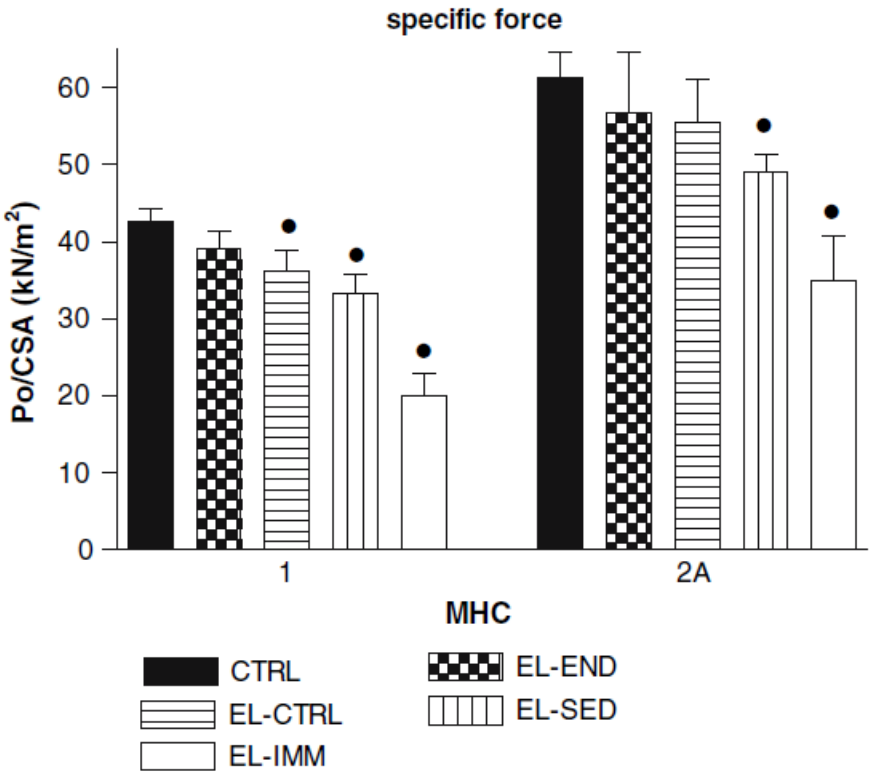


Figure 1.7. Age-related, skinned fibre specific force loss is ameliorated by physical activity.
Specific force from MHC I fibres (left) and MHC IIA fibres (right), obtained from young controls (CTRL), elderly endurance trained (EL-END), elderly controls (EL-CTRL), elderly sedentary (EL-SED), and elderly immobilized subjects (EL-IMM). Data are means ± SEM and specific force significantly ($p < 0.05$) different from the young control group is marked using the black dot. Specific force was not significantly different in elderly endurance trained individuals compared to young controls in either MHC I or IIA fibres. No significant difference was observed in MHC IIA fibre specific force from elderly control subjects compared to young controls. Otherwise, MHC I and MHC IIA fibre specific force was significantly lower in all other groups compared to young controls. These data suggest that physical activity prevents the onset of specific force loss in elderly individuals at the level of the myofilaments (D'Antona et al., 2007).

Results from some studies contradict the hypothesis that maintenance of physical activity in old age will maintain skeletal muscle fibre specific force. Frontera et al. (2000a) estimated the average weekly energy expenditure of their participants using a questionnaire, which revealed that the elderly men (n = 12; mean age: 74) averaged 1,431 ± 1,455 kcal/wk compared with 657 ± 747 kcal/wk (means ± SD) in the young men (n = 7; mean age: 37). Even though their average weekly energy expenditure was double that of the young men, specific force was significantly lower in MHC I and IIA skinned Vastus Lateralis fibres from elderly men compared with young men. However, the large standard deviation in physical activity levels for both young and elderly groups suggests that the physical activity profiles differed considerably between individuals in each experimental group. Therefore, the findings are unlikely to reflect the impact of physical activity on skinned fibre specific force.

MHC I and IIA Vastus Lateralis fibres from elderly individuals (n = 2; age range: 73-81) who had maintained an exercise programme for at least three to six days per week produced similar specific force to elderly controls (n = 2; age range: 73-81) and significantly less than young subjects (n = 4; 25-31) (Larsson et al., 1997). This suggests that physical activity does not prevent age induced specific force loss, although a weakness of this study is the low number of participants studied. A more strongly powered study of similar design measured specific force from Vastus Lateralis fibres of young adults (n = 6; mean age: 23), non-athlete elderly adults (n = 5; mean age: 78) and elite masters track and field athletes (n = 6; mean age: 79) (Power et al., 2016). P_0 and specific force were significantly higher in the young group compared with both elderly groups, while no significant differences were observed between the elderly groups, consistent with the findings of (Larsson et al., 1997).

1.7.4 Skinned fibre specific force measured from mobility-limited elderly cohorts

Supporting that the level of physical activity maintained in old age does not affect skinned fibre specific force, some research also suggests that muscle disuse does not have a negative impact on skinned fibre contractile properties. For instance, specific force, P_0 and CSA of MHC I and IIA Vastus Lateralis fibres were similar between elderly male (*n* = 12; mean age: 79) and female (*n* = 13; mean age: 77) subjects classified as mobility-limited based on the Short Physical Performance Battery test, healthy elderly males (*n* = 16; mean age: 74) and females (*n* = 7; mean age: 74), and healthy, middle aged males (*n* = 12; mean age: 47) and females (*n* = 11; mean age: 48) (Reid et al., 2012).

Interestingly, Venturelli et al. (2015) compared specific force from two muscles which in theory, undergo different degrees of disuse with advancing age, by comparing Vastus Lateralis and Biceps Brachii skinned fibre specific force. As age-related declines in physical activity are primarily associated with lower limb disuse, the Vastus Lateralis was expected to have undergone a greater degree of disuse than the Biceps Brachii, relative to the same muscles from young individuals. Vastus Lateralis and Biceps Brachii MHC I and IIA specific force was similar between healthy, young women (*n* = 8; mean age: 25), elderly mobile women (*n* = 8; mean age: 87) and elderly immobilised women (*n* = 8; mean age: 88) who had been wheelchair bound for two years and were unable to walk independently (Venturelli et al., 2015). These observations that mobility-limited and wheel chair bound elderly individuals do not exhibit specific force loss are surprising, given the reports in young cohorts that immobilisation causes a reduction in skinned fibre specific force (Trappe et al., 2008, Trappe et al., 2007, Larsson et al., 1996).

1.7.5 Observations of improved skinned fibre specific force with advancing age

Different still to the findings previously presented, recent research has suggested that the specific force generating capacity of Vastus Lateralis fibres improves with advancing age. In a three year follow up study on a mobility-limited elderly cohort (*n* = 22; mean age: 77) and healthy elderly cohort (*n* = 26; mean age: 74) MHC I and IIA Vastus Lateralis fibre specific force was significantly higher in both groups (Reid et al., 2014) compared with previously reported measurements (Reid et al., 2012). Consistent with these findings, Grosicki et al. (2016) reported Vastus Lateralis MHC IIA fibre specific force in eighty-eight year old men and women which was 42% greater than twenty year olds and 19% greater than younger octogenarians previously studied in their lab. MHC I fibre specific force was 31% greater in the eighty-eight year olds compared with the healthy twenty year olds. The authors attribute these findings to a maintenance of the highest quality muscle fibres in very elderly people.

1.7.6 Potential causes of the equivocal findings reported as to the occurrence of age-related specific force loss in skinned fibres

The equivocal results reported as to the occurrence of age-related specific force loss at the skinned fibre level are epitomised by the report of two different conclusions from skinned fibres form the same individuals, in the same study. Elderly men and women (*n* = 16; mean age: 71) who suffered from knee osteoarthritis, which substantially reduces weight bearing activity on the affected limb, exhibited similar MHC I and IIA Vastus Lateralis fibre specific force measured at 25°C, compared to healthy controls (*n* = 15; mean age: 68). However, MHC IIA specific force measured at 15°C, a lower experimental temperature, was significantly lower in the osteoarthritic group compared with healthy controls (Callahan et al., 2014). This suggests that experimental conditions are a factor which influence the results from experiments on skinned fibres.

Another example of a methodological factor affecting the conclusion from a given study was demonstrated by Toth et al. (2012). No change in MHC I and IIA Vastus Lateralis fibre specific force measured at both 15°C and 25°C was observed in sedentary, elderly men and women (n = 14; mean age: 72) as a result of an eighteen week resistance training programme. Electron microscopy (EM) images revealed that the myofibril area fraction was reduced following resistance training. Therefore, P_0 was normalised to the myofibril area (~74-84% of CSA) as opposed to the CSA, which revealed that skinned fibre specific force measured at 25°C was significantly increased in MHC I and IIA fibres following resistance training. The authors suggest that skinned fibre CSA may not be stable proxy of the area of contractile machinery. Indeed, this may be the case, given the variable change in skinned fibre CSA which occurs as a result of swelling due to the chemically skinned procedure (discussed in section 2.1.6). However, these data must be interpreted with caution because the myofibrillar fraction was extrapolated using the EM data from a sub-set of the participants studied.

If skinned fibre CSA does not accurately represent the myofibrillar proteins contributing to force generation, then this could explain why shorter duration (twelve week) resistance training studies have not reported increased specific force. However, Toth et al. (2012) only observed a different trend when normalising force data obtained at 25°C, not 15°C, to the myofibrillar fractional area. Based on the fact that the other studies considered thus far were conducted at 12°C or 15°C, normalising P_0 to myofibrillar fractional area may make no difference.

Relatively few studies (Larsson et al., 1996, Trappe et al., 2003, D'Antona et al., 2003) have attempted to quantify the myofibrillar protein content of the elderly and young cohorts used to study the onset of specific force loss. This is likely due to the numerous technical considerations (outlined in sections 3.12 and 5.3.4) which must be taken into

account when quantifying protein content using Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE), one of the most commonly employed methods (Geiger et al., 2000). Given that the skinned fibre preparation is used to represent the interaction of the myofilaments during a muscle contraction, the myofibrillar protein content is one of the primary factors influencing skinned fibre specific force. Therefore, information about the myofibrillar protein content in a given experimental group could provide important information regarding the trends in reported results (discussed in section 1.7.7.1).

1.7.7 Mechanisms of human skinned fibre specific force loss

Published reports have shown that physical activity maintains specific force and immobilisation causes a decrease in specific force (D'Antona et al., 2007), while other studies observed a lower specific force in master athletes (Power et al., 2016), or no change following prolonged muscle disuse (Venturelli et al., 2015). Contrary to all the aforementioned trends, increases in specific force have been observed with advancing age (Grosicki et al., 2016). Clearly, even when results are considered in the context of a given physical activity profile of the cohorts studied, considerable disparity is observed. However, despite the equivocal results regarding a) the occurrence of specific force loss in sedentary elderly people, b) the maintenance of specific force by maintaining physical activity and c) whether elderly people are actually left with fibres which are capable of producing higher specific force values than young people, the mechanism of specific force production with regard to the myofibrillar proteins is relatively consistent. The following section discusses the published findings regarding the association between myofibrillar protein and specific force production.

1.7.7.1 The association between skinned fibre myofibrillar protein content and specific force

The generally accepted view that the observation of age-related specific force loss is inconsistent in the skinned fibre literature is summed up by D'Antona et al. (2007):

"Notwithstanding the large body of information on single muscle fibre contractile properties in old age, contradictory results have still been reported in the two most recent and detailed studies performed so far (D'Antona et al., 2003) (Trappe et al., 2003)."

However, while some studies have reported reduced specific force in elderly individuals and others have not, the reported mechanism regulating specific force with regard to skinned fibre myofibrillar protein content appears to be consistent.

Skinned skeletal muscle fibre myosin concentration has been quantified by measuring the optical density of a given fibre's MHC bands on SDS PAGE. A lower myosin concentration in Vastus Lateralis fibres from young men (n = 3; mean age: 28) was associated with reduced specific force, as a result of thirty-seven days of bed rest, although the myosin concentrations of the different fibre types were not specified (Larsson et al., 1996). D'Antona et al. (2003) correlated the myosin concentration of Vastus Lateralis fibres from young (n = 7; mean age: 30), healthy elderly (n = 7; mean age: 73) and immobilised elderly (n = 2; age 70 and 72) men with specific force measured from a separate set of fibres from the same individuals. The myosin content of each group displayed a positive linear relationship with skinned fibre specific force, meaning skinned fibres exhibiting the lowest or highest specific force also had the lowest and highest myosin concentrations respectively. These findings clearly demonstrated that specific force loss is associated with a reduced myosin content.

In contrast to the aforementioned findings, no difference in myosin and actin concentrations in homogenate muscle samples were observed between life-long sedentary young (n = 6; mean age: 25) and elderly (n = 6; mean age: 80) men, or between young (n

= 6; mean age: 25) and elderly (*n* = 6; mean age: 78) women. The lack of difference in myofibrillar protein concentration mirrored the lack of a difference in skinned fibre specific force between the young and elderly groups (Trappe et al., 2003). Furthermore, in middle aged (*n* = 8; mean age: 43) individuals who underwent seventeen days of bed rest, no change in Soleus myosin thick filament density occurred, as indicated by electron microscopy (EM) images (Widrick et al., 1997). The lack of difference in myosin thick filament density pre- and post-bed rest was associated with no difference in specific force.

The relationship between skinned fibre specific force, myofibrillar protein content and cross-bridge behaviour, has been examined in healthy individuals and compared to models of specific force loss in pathological conditions other than ageing. Ochala and Larsson (2008) observed significantly lower specific force in MHC I and IIA Vastus Lateralis fibres from a group of three patients suffering from acute quadriplegic myopathy (AQM) and one from small cell lung cancer (*n* = 4; age range: 22-77) compared with healthy age matched controls (*n* = 7; age range: 26-67). The specific force loss was associated with a reduction in the ratio of myosin:actin, indicating a preferential degradation of myosin in the patients compared to the healthy controls. Furthermore, when specific force was divided by the ratio of myosin:actin, no differences in specific force per unit of myosin/actin was observed between either group in MHC I and IIA fibres. This supports that skinned fibre specific force is modulated by myofibrillar protein content (Ochala and Larsson, 2008).

The aforementioned studies indicate that there is a strong relationship between myofibrillar protein content and the intrinsic force generating capacity of skinned fibres, irrespective of whether specific force loss occurs in one group relative to another. Therefore, data quantifying the skinned fibre protein content provide a mechanistic basis to support the observed trends in specific force between groups. Some studies indicate

that a reduced myosin content can be caused by physical inactivity such as cast immobilisation (D'Antona et al., 2003) or thirty-seven days of bed rest (Larsson et al., 1996), whereas others have not observed reductions in myosin content or specific force due to inactivity of shorter duration, i.e. seventeen days of bed rest (Widrick et al., 1997).

1.7.7.2 The relationship between protein content and instantaneous stiffness in human skinned fibres

The 'stiffness' of a muscle fibre is used to reflect the number of bound acto-myosin cross-bridges during an isometric contraction, and refers to the dependence of P_0 on fibre length at a given moment (Huxley and Simmons, 1971). Furthermore, evidence indicates that cross-bridges can be bound in high force or low force producing states (Lowe et al., 2001, Ostap et al., 1995), indicating that the behaviour, as well as the number of cross-bridges, can influence force production.

The association between reduced skinned fibre specific force and myofibrillar protein content suggests that the mechanism of specific force loss is a lower number of available cross-bridges during an isometric contraction. Indeed, skinned fibre stiffness, representing the number of bound acto-myosin cross-bridges during an isometric contraction, was significantly lower in both MHC I and IIA fibres from AQM patients compared with healthy, control participants. The reduced stiffness corresponded to the reduction in specific force and myosin in the AQM patients (Ochala and Larsson, 2008).

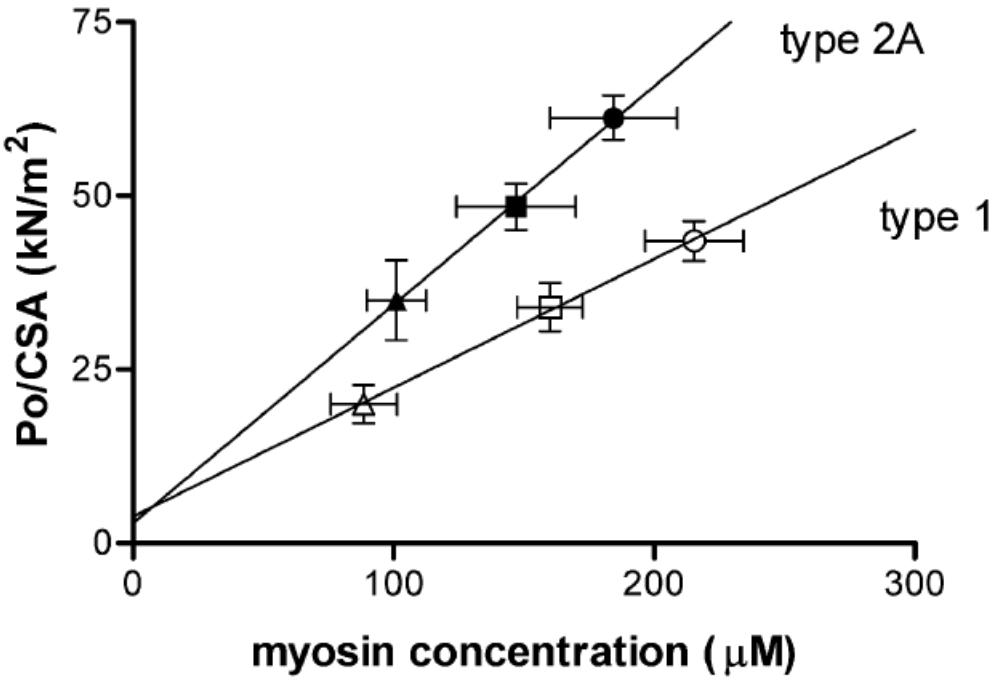


Figure 1.8. The relation between skinned fibre specific force and myosin concentration.

Myosin concentration and specific force of MHC I (clear symbols) and IIA (filled symbols) single skinned fibres from young individuals (circles) (specific force measurements: MHC I, *n* = 60; MHC IIA, *n* = 45), sedentary elderly individuals (squares) (specific force measurements: MHC I, *n* = 49; MHC IIA, *n* = 28) and elderly individuals whose leg had been immobilized for 3.5 months in a cast (triangles) (specific force measurements: MHC I, *n* = 28; MHC IIA, *n* = 21). A clear linear relationship between skinned fibre specific force and myosin content was observed between the experimental groups studied. The sloe of the regression lines for MHC I (0.185 ± 0.006) and MHC IIA (0.314 ± 0.41) were both statistically different from zero ($p < 0.05$). This relationship suggests that skinned fibre specific force is strongly related to myosin content.

A strong linear relationship was observed between relative stiffness and relative force for both MHC I and IIA fibres from both groups. This indicates that skinned fibre force is proportional to the percentage of available cross-bridges which are bound during an isometric contraction in both patients and healthy controls. Furthermore, the ratio of specific force:fibre stiffness at a given pCa was similar between healthy controls and patients, suggesting that the force produced by each cross-bridge was similar between the two groups (Ochala and Larsson, 2008). Collectively, these findings support that skinned fibre specific force is modulated by the number of bound acto-myosin cross-bridges, which are in turn modulated by the concentration of myofibrillar protein in a given skinned fibre.

1.7.7.3 Evidence that specific force is affected by cross-bridge behaviour

Evidence suggests that even when there is no reduction in skinned fibre myofibrillar protein content, cross-bridge behaviour, as opposed to the number of bound cross-bridges, can reduce force production (Toth et al., 2013). Myosin and actin content in cancer patients (n = 11; mean age: 59), quantified from homogenate muscle samples run on SDS PAGE gels, was similar to age matched healthy controls (n = 6; mean age: 65). Accordingly, MHC I Vastus Lateralis fibre specific force did not differ between groups, but MHC IIA fibre specific force was 14% lower in cancer patients despite the lack of difference in myofibrillar protein content. This was attributed to a significantly reduced number of bound cross-bridges in MHC IIA fibres from cancer patients, as indicated by stiffness measurements (Toth et al., 2013). When considered in the context of ageing, these results suggest that specific force loss could occur due to a lower number of bound cross-bridges, even when myofibrillar protein content is not diminished.

Interestingly, some evidence exists to suggest that acto-myosin cross-bridge behaviour can, in fact, compensate for a lower myofibrillar protein concentration i.e. fewer total cross-bridges available, in order to maintain specific force. MHC I Vastus Lateralis fibres from a mixed-sex cohort of heart failure patients (n = 10; mean age: 72) displayed lower specific force, lower myosin content and subsequently lower acto-myosin cross-bridge stiffness compared to age matched controls with a similar daily physical activity profile (n = 10; mean age: 69) (Miller et al., 2009). Thus, the results for type I fibres were consistent with the idea that reduced skinned fibre specific force was due to a lower total number of cross-bridges. In contrast, MHC IIA fibre specific force and fibre stiffness was not significantly different in heart failure patients despite a significantly lower myosin content compared to young controls. The maintenance of fibre stiffness was explained by a higher proportion of the available cross-bridges being in a bound state, as indicated by the higher ratio of fibre stiffness to in rigor fibre stiffness, the latter representing the total cross-bridges available in a given skinned fibre (Miller et al., 2009). Therefore, although outside the context of ageing, these results suggest that acto-myosin cross-bridge behaviour can compensate for reduced myofibrillar protein content.

1.7.7.4 Evidence that specific force is affected by the force producing 'state' of an actomyosin cross-bridge

In the context of human ageing, some evidence suggests that there is an alteration in the force producing 'state' of bound acto-myosin cross-bridges which causes a reduced specific force in elderly compared to young individuals. Ochala et al. (2007) calculated the ratio of skinned fibre stiffness and specific force (K/SF) to provide an indication of the cross-bridge stiffness per unit of force. K/SF was significantly higher in MHC I and IIA Vastus Lateralis fibres from elderly men (n = 6; mean age: 66) compared with young

men (*n* = 6; mean age: 32), despite the fact that MHC I and IIA specific force was significantly lower in elderly men. Skinned fibre myosin content was not quantified, but the results suggest that there are a higher number of acto-myosin cross-bridges bound in a low force state in skinned fibres from elderly men, thus increasing the fibre stiffness in the presence of reduced specific force (Ochala et al., 2007). These findings indicate that specific force can be modulated both by the number and behaviour of bound acto-myosin cross-bridges.

That cross-bridges can be in low-force generating states is supported by more direct evidence from rodent models of ageing. Lowe et al. (2001) observed a 27% reduction in specific force produced by small fibre bundles from elderly rats (*n* = 6; age: 32-36 months) compared to young rats (*n* = 6; age: 8-12 months), which corresponded to a 30% reduction in myosin heads attached in a strong-binding structural state, as indicated by electron paramagnetic resonance.

1.7.7.5 Other proposed mechanisms of age-related specific force loss

The simplest explanation for a reduction in specific force would be an impaired ability of elderly individuals to fully activate their muscles, either due to poor motivation or reflex inhibition. This would indicate that for a given amount of muscle mass, fewer individual fibres would be recruited, resulting in reduced force output. However, Phillips et al. (1992) used electrical stimulation on the Adductor Pollicis muscle in elderly subjects who exhibited specific force loss to test whether they could fully activate their muscles, and found that they could.

At the molecular level, Thompson et al. (2006) demonstrated that MHC content was significantly reduced with age but actin content remained the same in the Semi-membranosus muscle of rats. The preferential reduction in myosin content may alter the

stoichiometry of the myofibrillar proteins relative to each other, contributing to the reduction in specific force. Furthermore, alterations in the myosin molecule may occur via non-enzymatic glycosylation, as myosin contains several potential targets for glycation (Ramamurthy et al., 2001). Indeed, an 18-25% ageing related slowing was observed in the speeds at which actin filaments were propelled (V_f) by the MHC I myosin isoform in an in vitro motility assay (IVMA) preparation of rat soleus muscle fibres (Hook et al., 2001).

In humans, significant reductions in both MHC I and IIA motility speeds which were associated with post-translational modifications of the myosin molecule were observed in IVMA preparations of fibres from elderly compared to young individuals. Furthermore, a higher disorder of myosin molecules during isometric contraction in skinned fibres was observed, based on the lower intensity of the low angle X-ray diffraction reflection (MM1 reflection). The MM1 reflection corresponds to the 14.3nm axial repeat of the myosin filament head and an increase in reflection intensity is indicative of a higher order in the arrangement of the myosin heads, discussed further in Chapter 5 (Li et al., 2015). The aforementioned findings suggest that kinetic changes in the myosin molecule may affect interaction with actin and subsequent formation and force production of cross-bridges.

Canepari et al. (2005) did not observe a clear effect of ageing on V_f in myosin isoforms extracted from MHC I and IIA Vastus Lateralis fibres from young and elderly individuals. Interestingly, an increase in V_f was observed in MHC IIA isoforms following resistance training in both young and elderly groups, suggesting a positive effect of exercise on the motility speed of fast myosin isoforms. However, the rate of cross-bridge cycling as measured by a slacken-restretch procedure was found to be lower in skinned fibres from non-athlete and master athlete elderly individuals compared to young, healthy

controls. These findings suggest that an age-related slowing of cross-bridge kinetics occurs, irrespective of exercise (Power et al., 2016).

1.7.7.6 Summary of mechanisms

Evidence suggests that skinned skeletal muscle fibre specific force is modulated by myosin concentration, with low and high myosin concentrations associated with low and high specific force values respectively (D'Antona et al., 2003). The number of bound acto-myosin cross-bridges during an isometric contraction, which is strongly related to the specific force generated, has been shown to be largely dependent on the skinned fibre myofibrillar protein content (Ochala and Larsson, 2008). Evidence also indicates that cross-bridges can be bound in a low force producing state (Ochala et al., 2007, Lowe et al., 2001), or can compensate for a lower myosin concentration (Miller et al., 2009). Therefore, cross-bridge behaviour affecting the force produced per cross-bridge, as well as the number of bound cross-bridges, has been shown to affect skinned fibre specific force.

Given the equivocal results which have been previously discussed (sections 1.7.2, 1.7.3, 1.7.4, and 1.7.5), it is not possible to draw firm conclusions as to whether age-related specific force loss occurs at the skinned fibre level. The most reliable conclusions that can be drawn are from data which are supported by measurements of myofibrillar protein, since specific force and the occurrence of specific force loss has consistently been observed to be related to myofibrillar protein content (D'Antona et al., 2003, Trappe et al., 2003). However, even within these studies, differences in observations of whether age-related specific force loss occurs suggest that factors determining myofibrillar protein content are more important than chronological age, for the development of skinned fibre specific force loss.

1.7.8 Power measured from chemically skinned skeletal muscle fibres

Studies investigating the power output of skinned fibres have demonstrated that MHC IIX fibres produce the highest power output, followed by MHC IIA fibres, with MHC I fibres exhibiting the lowest power output (Widrick et al., 1996b). The interaction of P_0 and V_{max}, as described by the force-velocity curve, results in the generation of power, which is how muscle fibres would be expected to behave *in vivo*. Few studies have reported power from elderly individuals compared to young cohorts (Trappe et al., 2003, Reid et al., 2014, Reid et al., 2012, Callahan et al., 2014, Grosicki et al., 2016). Any differences reported between groups are dependent on differences in P_0 and/or V_{max}, which have been discussed previously. Reductions in skinned fibre peak or normalised power in response to different interventions have been attributed to decreased P_0 in several investigations (Widrick et al., 1999, Widrick et al., 1998, Widrick et al., 2001). This supports that identifying the onset and mechanism of skinned fibre force loss is important in elucidating the cause of reductions in skinned fibre power which may be prevalent *in vivo*.

1.8 Summary and aims of the present thesis

The literature review aimed to introduce the concept of age-related skeletal muscle weakness and highlight the fact that muscle quality, as opposed to quantity, is a prevalent factor causing the decline in muscle strength with advancing age. The importance of recruiting physically active, healthy elderly individuals for research investigating age-related specific force loss was discussed, and the chemically skinned fibre preparation was introduced as a means of probing the mechanism of age-related specific force loss. Given the equivocal findings from experiments studying skinned fibres from young and elderly cohorts, and the proposed mechanisms of age-related specific force loss at the level of the myofilaments, the aims of the present thesis were to:

1. Systematically investigate the variation in skinned fibre specific force values reported in the literature, identify causative factors and where possible, quantify their contribution to the variability in published specific force values.
2. Address the impact of using different activating solutions to elicit force from chemically skinned skeletal muscle fibres on the variability in reported specific force values, as well as the equivocal conclusions drawn by different studies as to the occurrence of age-related specific force loss at the level of the myofilaments.
3. To measure and compare skinned fibre specific force between a carefully characterised cohort of physically active master cyclists, comparatively frail hip fracture patients, and a healthy, young control group. The mechanism of specific force with regard to skinned fibre myosin content was assessed and potential differences in cross-bridge behaviour between the young and elderly cohorts were probed by comparing specific force results from two different activating solutions, and from low-angle x-ray diffraction patterns of the myosin meridional reflection.

2 Addressing the variance in specific force measurements from single, chemically skinned human muscle fibres: a systematic review

2.1 Introduction

In human skeletal muscle, assessing specific force *in vivo* is complicated by a number of factors, which relate to both the measurement of force and cross-sectional area, as outlined in section 1.5 (Haxton, 1944, O'Brien et al., 2010). Attempts to address the limitations of *in vivo* approaches have been made using the chemically skinned fibre technique *in vitro*, in order to study human muscle function and specific force (section 1.6).

Despite the advantages of being able to study the contractile properties of a single skeletal muscle fibre, there appears to be considerable variation in human skinned fibre specific force reported by different publications. This observation contradicts the well accepted idea that a reliable experimental technique will yield reproducible results across different investigations. The variability in reported skinned fibre specific force values makes comparisons of results from different studies difficult and raises questions as to the validity of the skinned fibre technique with regard to reflecting *in vivo* function. Therefore, the aims of this chapter were to:

1. Systematically evaluate the degree of variability in published specific force values from human, skinned skeletal muscle fibres.
2. Identify factors causing the variation in published results.
3. Determine the contribution of any identified factors to the variation in published results.

2.2 Methods

A systematic search of the human skinned skeletal muscle fibre literature was undertaken and methodological differences between studies which may cause the variation in reported specific force results were identified. The effect of each of the identified methodological factors was quantified. The analysis of specific force was performed on data obtained from control or pre-intervention groups, in order to exclude the effects of disease, any interventions and the effects of ageing, thereby enabling the comparison of results from different studies. Analysis was constrained to fibres containing MHC I and MHC IIA isoforms, excluding MHC-IIX and hybrid fibres.

2.2.1 Search strategy

A systematic search was carried out in 'Medline,' 'Embase Classic + Embase' and 'Web of Science.' Each database was searched from the earliest to most recent year or week of publication available. Criteria adapted from the Cochrane Collaboration guidelines for conducting a systematic search were used, which included consulting an IT specialist who had expertise in systematic review methodology, in order to optimise the search strategy. Once the search had been conducted (June, 2014) the 1,876 search results were compiled into an EndNote reference library. From this library, two people independently selected studies to be included in the review and compared their decisions, in order to decrease the probability of omitting relevant papers.

The inclusion criteria were: (1) published in English; (2) using chemically skinned fibres from the muscle biopsies of human lower limb muscles; (3) reporting measurements of skinned fibre specific force and/or both maximal isometric force and cross-sectional area; (4) having a control or pre-study intervention group; (5) having a subject group not consisting solely of highly trained athletes; (6) subjects included males

and/or females under 45 years old. Some studies which contained groups of older or unspecified age were also included, discussed in section 2.2.9. A total of 73 studies met the inclusion criteria and were used for a meta-analysis. Their dates of publication span the period 1989 to 2013, but only 3 studies were published before 1996.

Specific force and, where available, fibre peak isometric force (P_0) and cross-sectional area (CSA) data were extracted from each publication, using pre-intervention or control groups where appropriate, thus eliminating the impact of any training or other intervention effect. Data were taken from numerical tables where possible. When numeric data were not available, values were measured from enlarged versions of the figures using purpose written MatLab software. Professor Roger Woledge wrote all Matlab software used in this study. A second investigator cross checked the compiled data against the original source to minimise the chance of errors occurring.

2.2.2 Data collection, sub-grouping and assessment of variability

The 73 publications which met the inclusion criteria were objectively divided into research groups based on shared authorship using a purpose written Matlab programme. Many of the 73 publications included several sets of data which corresponded to specific force measured from different fibre types, skeletal muscles, experimental groups composed of males, females or both genders, etc. 153 sets of specific force data were obtained, of which 132 also had corresponding CSA and/or P_0 data. Where only CSA *or* P_0 was provided, the missing measurement was calculated using the reported specific force value. The 153 data sets were divided into 3 groups based on the temperatures at which the experiments were conducted: 12^0C, 15^0C and $>18^0$C. The relative numbers of data sets in each temperature group are shown in Table 1.

The compiled specific force data were plotted by temperature group in Forest Plots to observe the heterogeneity in reported values for MHC I and IIA fibres within each temperature range. A relatively large and relatively homogenous group of data (the 15^0C group) was used for examining the influence of specific methodological factors. The data for MHC I and MHC IIA fibres were initially kept separate, but were combined later during the analysis.

2.2.3 Adjustments made to published data based on methodological differences between studies

The variability in reported specific force data was assessed using scatter diagrams of the mean P_0 against the mean CSA reported by a given study, for both MHC I and MHC IIA fibres. The effect of methodological differences between studies was assessed by adjusting the individual P_0 or CSA values from the compiled data sets for a given methodological difference, re-plotting the scatter diagrams using the adjusted data and observing any change in the P_0 vs CSA relation. How the adjustments for each methodological difference were made is described below, and the impact of each adjustment on the variance in the data is summarised in Table 2.2. The combined effect of all the adjustments for methodological differences on the dispersion of the data is illustrated in the Figure 2.3E and F for MHC I and MHC IIA fibres respectively.

2.2.3.1 Adjustment for skinned fibre swelling

Some, but not all authors "corrected" their specific force measurements either by multiplying the specific force by 1.44 ($=1.2^2$) or 1.56 ($=1/0.8^2$), to account for the swelling which increases a fibre's CSA and occurs as a result of chemical skinning. 56 of the 73 publications specified whether or not they had adjusted their skinned fibre CSA values

for swelling. To examine any effect on the dispersion of the data, the correction for swelling was removed and the P_0 vs CSA relationship was replotted.

17 of the 73 studies did not clearly state whether their reported mean skinned fibre CSA measurement had been adjusted by a factor of ~1.5, to account for the reported 20% increase in fibre diameter which occurs due to fibre skinning. Therefore, determining which publications reported specific force when fibre swelling had or had not been taken into account needed to be clarified. In most cases, whether or not CSA values had been corrected for swelling could be determined by calculating CSA using the corresponding P_0 and specific force measurements. The reported CSAs agreed with the calculated CSAs in three publications. The reported CSAs were ~1.5 times greater than the calculated CSAs for 7 publications, so in these cases the reported CSA values were "uncorrected" for swelling. 7 publications remained which reported mean CSA and specific force were reported, but not mean P_0. Therefore, one author from each of the remaining 7 publications was contacted for clarification, the conclusion being that all 7 papers reported CSAs corrected for fibre swelling. In one publication (Larsson and Moss, 1993) the force values in the tables are ten times the likely values, which was assumed to be a typographic error and corrected. Overall, 35 of the 153 data sets, mostly in the 15°C group, had corrected for swelling, so an adjustment was applied to undo this 'correction.'

2.2.3.2 Different CSA measurement techniques

Three different methods had been used for measuring CSA:

CSA_E: Measuring the major ('width') and minor ('depth') axes of the fibre and assuming an elliptical cross section. 64 of the 153 data sets used this method.

CSA_C: Measuring only the width of the fibre and calculating CSA assuming a circular cross section. with the width value used as diameter. Whether the 'width' represents the

major axis of the generally elliptical shape of the fibre, or an average of the minor and major axes is not clear with this method. 39 of the 153 data sets used this method.

CSA_A: Measuring the fibre diameter while the fibre is suspended in air, and thus presumably compressed to a circular shape by surface tension, and assuming a circular cross section. 50 of the 153 data sets used this method.

The three aforementioned CSA calculation methods have been compared by Degens and Larsson (2007) who show that:

$CSA_E = Q^{AE} * CSA_A$ and $CSA_E = Q^{EC} * CSA_C$

Where Q^{AE} = ~0.7 and Q^{EC} = ~0.4

Several other authors using method CSA_E have noted the aspect ratio of the fibres they used, and the values are generally larger than in the experiments of Degens and Larsson's (2007) experiments. Therefore when considering all of the included studies together the values of Q^{EC} and probably also Q^{AE} should be less than Degens and Larsson's (2007) values. To find how these different methods might have caused dispersion among the data, CSA_A and CSA_C values were converted to CSA_E using Q values chosen to maximise the correlation between P_0 and CSA for the 15^0C data.

2.2.3.3 Adjusting data based on sarcomere length

An optimal sarcomere length (SL) of 2.75µm yields maximum P_0 development in human skinned fibres (Gollapudi and Lin, 2009). However, 54% of the studies included in this systematic review used a sarcomere length of 2.5µm, while other studies used SLs ranging from 2.1-2.8µm. Altering SL affects both P_0 and CSA of skinned fibres, as is described below.

P_0 falls to 76% of maximum in an essentially linear manner in the SL range 2.75-2.1µm as shown by the red line in Figure 2.4. The reported data from each study were adjusted to represent the specific force which would have been obtained had an optimal SL been used, by using the previously published data on the force-SL relationship in human skinned fibres (Gollapudi and Lin, 2009).

Published data on the effect of skinned fibre CSA as a function of SL could not be found, and whether skinned fibres exhibit constant volume behaviour is not known. The *filament lattice* of mechanically skinned frog single fibres did not display constant volume behaviour, but CSA decreased as the fibres were stretched, more so when in propionate solution than in chloride solution, as indicated by the dashed and dotted green lines in Figure 2.4 (Matsubara and Elliott, 1972). In contrast, the filament lattice of skinned crayfish muscle displayed constant volume behaviour on stretching (April and Brandt, 1973). If, as in intact fibres, skinned fibres maintain a constant volume, CSA would vary as the reciprocal of SL (full black line in Figure 2.4). Since the filament lattice occupies ~80% of the volume of muscle fibres, the different SLs used by different studies could be accounted for by adjusting the reported skinned fibre CSA. However, applying several corrections to the compiled data sets, up to the full constant volume factor, yielded no improvement in correlation between P_0 and CSA. Therefore, an adjustment of CSA based on SL was not implemented in the present investigation.

2.2.4 Assessing variability within studies

In order to determine whether specific force measured using skinned fibres was consistent within studies, a paired comparison was made between MHC IIA fibre and MHC I fibre specific force using both a scatter diagram Figure 2.5A and a Bland Altman plot (Figure 2.5B). 60 of the 73 publications reported specific force values for both of

these fibre types. This comparison would also reveal whether the specific force of MHC I and MHC IIA fibres is consistently found to be different. Furthermore, using the mean difference between MHC I to MHC IIA specific forces, the P_0 vs CSA relationships from different fibre types could be combined by multiplying the reported MHC I specific force values by 1.19.

2.2.5 Adjustment for temperature differences

Four publications (Bottinelli et al., 1996, Stienen et al., 1996, Brenner et al., 2012, Godt and Lindley, 1982) report P_0 measured from skinned fibres at two or more temperatures, which when compared with force data measured at different temperatures in whole muscle (Ranatunga et al., 1987), shows that the average temperature sensitivity of human skinned fibres is similar to that *in vivo*. These data are plotted for comparison in Figure 2.6. The *in vivo* experiments provide a continuous function for the effect of temperature on specific force, which was used in the present study to adjust measurements from skinned fibres made at temperatures other than 15°C, to the expected values at 15°C. Thus, the specific force data from the three temperature groups could be combined, accounting for all previous adjustments (CSA swelling, CSA shape, SL, fibre type) made.

2.2.6 Differences in the activating solutions

Whether differences in the concentration of PCr, ATP, free Ca, pH buffer or ionic strength showed any correlations with reported specific force in the included studies was tested. There was only a trend between the concentration of PCr in the activating solution and the reported specific force, so this relationship was examined.

2.2.7 Date of publication

Whether there was a relation between a study's year of publication and the reported specific force was examined by relating the date of publication with the corrected specific force values.

2.2.8 Variability between research groups

An analysis of variance comparing the different publication groups was carried out to determine the probability that the groups' reported specific force values were from the same population.

2.2.9 Differences in subject populations

Publications which studied participants with a wide range of ages were included for analysis in the present investigation. To be certain that the inter publication group variability was not due to cohorts of different ages being studied by the different publications, the analyses were repeated excluding publications which studied participants over 40 years old or where the age was not specified. Studies in which biopsy samples were frozen in liquid Nitrogen before skinning were also excluded, for reasons which are described in the discussion. The total number of data sets included in the sub-analysis was reduced from 153 to 117.

2.2.10 Statistical analysis

A two-way ANOVA was used to assess whether skinned fibre specific force was significantly different between genders and/or different muscles. In order to ensure that any differences reported were not due to the fact that the data was measured by different research groups or at different experimental temperatures, comparisons were limited to specific force data which was measured by the same research group at the same temperature for a given fibre type (MHC I or IIA).

Forest Plots were made using OpenMeta freeware and accounted for the standard deviation (SD) of each included data set, as well as weighting individual data sets based on the *n* number of fibres of each data set. Regression lines relating P_0 and CSA were fitted with a Model II method (York et al., 2004). In Model II regressions, both CSA and P_0 are assumed to contain some measurement error.

Box and whisker plots were made using the MatLab boxplot function. The central line in each box represents the median value, and the lower and upper edges of each box represent the 25^{th} and 75^{th} percentiles respectively. The whiskers extend to the most extreme data points not considered outliers. Any specific force data approximately ± 2.7 times the standard deviation of the data was considered an outlier and plotted individually. Specifically, data points were considered outliers if they were larger than $q_3 + 1.5(q_3-q_1)$ or smaller than $q_1 - 1.5(q_3 - q_1)$, where q_1 and q_3 are the 25^{th} and 75^{th} percentiles, respectively. To compare results between different groups of sources the Kruskal-Wallis Anova (a non-parametric, rank order method) implemented in MatLab was used. Results are given as means ± SEM except where stated otherwise. Correlation values (R) are Pearson product moment values.

2.3 Results

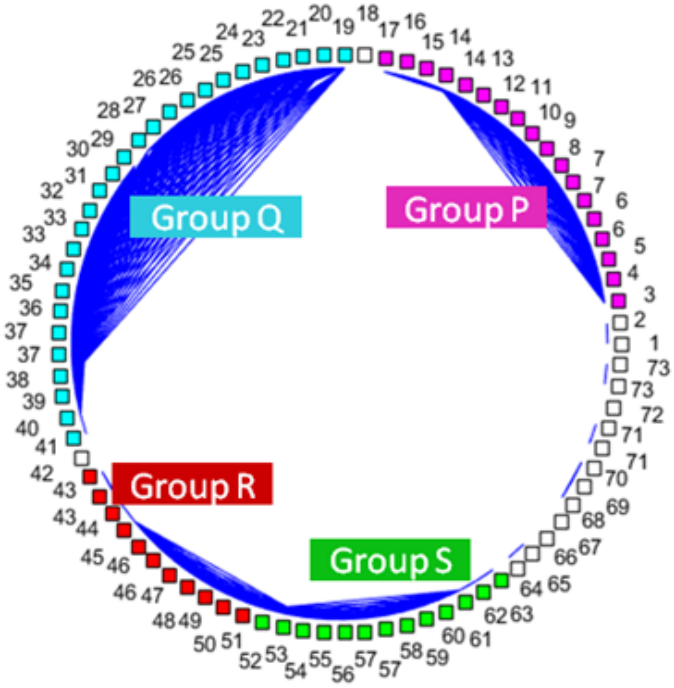
2.3.1 Objective grouping of publications

The 73 publications which met our inclusion criteria were objectively divided into research groups based on shared authorship. 59 of the 73 publications formed four research groups, exhibiting substantial shared authorship within each group and minimal shared authorship between groups (Figure 2.1). 14 of the 73 publications did not fall into any shared authorship grouping so were not included in the comparisons of research groups, but were included in all other analyses.

2.3.2 Variability in reported specific force

The two way ANOVA output indicated that specific force results measured from skinned fibres by the same research group at the same temperature were not significantly different ($p > 0.05$) between males and females or between skinned fibres of the same MHC isoform expression from different muscles. Therefore, all one 153 compiled data sets were used for further analysis.

All the forest plots of the MHC I and IIA data sets in each temperature group (Table 2.1) had a significant χ^2 ($p < 0.01$), indicating that there was heterogeneity present in all groups of compiled specific force data. This is illustrated in the Forest Plot in Figure 2.2, which displays the variability of specific force data measured at 15°C from MHC IIA fibres, the group of data which exhibited the lowest range of specific force. The I^2 value for this group of data was 98%, indicating that even in where the range of specific force values is lowest, the heterogeneity is still extremely high. The Forest Plots for all other groups of data sets are included in the section 8.2 of the appendices.



Publication group	*Source publications*
Group P:	(3) Pansarasa 2009; (4) Maffiuletti 2006; (5) Linari 2004; (6) Her 2000 (7) Harridge 1996; (8) DAntona 2007; (9) DAntona 2003; (10) DAntona 2006 (11) Bottinelli1999; (12) Bottinelli 1998 (13) Bottinelli1996 (14) Stienen 1996; (15) Szentesi 2005; (16) Szentesi 2001; (17) Lassche 2013
Group Q:	(19) Trappe 2008; (20) Trappe 2007; (21) Trappe 2004; (22) Trappe 2003 (23) Trappe 2006; (24) Raue 2009; (25) Luden 2008; (26) Luden 2012 (27) Harber 2004; (28) Harber 2008; (29) Widrick 2002a; (30) Shoepe 2003 (31) Garner 2003; (32) Choi 2010a; (33) Widrick2002b; (34) Widrick 2001 (35) Widrick 1999; (36) Widrick 1998; (37) Widrick 1997a; (38) Widrick 1996a (39) Widrick1997b; (40) Galler 1997; (41) Hilber 1997
Group R;	(43) Mounier 2009; (44) Pathare 2005; (45) Krivickas2002; (46) Reid 2012; (47) Krivickas 2000; (48) Frontera 2000; (49) Frontera 2003; (50) Ochala 2007a (51) Ochala 2006
Group S:	(52) Larsson 1997; (53) Larsson 1996; (54) Frontera 1997; (55) Ochala 2007b (56) Ochala 2008; (57) Yu 2007; (58) Larsson 1993; (59) Larsson 1995 (60)Korhonen 2006; (61) Degens 1999; (62) Gilliver 2009; (63) Eskine 2011
Ungrouped sources:	(1) Tavernier 1996; (2) Adnet 1996; (18)Campbell 1999; (42) Claflin 2011 (64) Hvid 2011; (65) Hvid 2013; (66) Kohn 2013; (67) Malinchik1997 (68) Malisoux 2007; (69) Malisoux 2006a; (70) Malisoux 2006b; (71) Miller 2013; (72) Ruff 1989; (73) Seebohm 2009

Figure 2.1. Publication groups formed based on shared authorship.
Each box around the circle represents one of the 73 publications included in the systematic review. A blue line between two boxes indicates that a given pair of publications shared one or more authors. The publications are arranged around the circle in an order that minimizes the crossover of the blue lines. The different publication groups are illustrated by the different coloured markers and have been named group P, Q, R or S. Publications which did not form a publication group are indicated by the white squares, and their results are included in all analyses except for those comparing publication groups.

Temperature group	12^{0}C	15^{0}C	>18^{0}C
Numbers of data sets providing values for specific force			
Type 1	20	44	16
Type 2A	18	38	15
Total	38	82	31
Numbers of data sets providing corresponding force and area measures			
Type 1	16	45	10
Type 2A	14	38	9
Total	30	83	19

Table 2.1. Numbers of data sets which contributed to the analyses displayed Figure 2.3 and in Table 2.2.

The box and whisker plots in Figure 2.3 represent the range of reported specific force values in each of the 3 temperature groups. Even after eliminating outliers the range of values was substantial, exhibiting a high degree of variability in published specific force values. The largest variation was more than 8-fold for MHC I fibres at 12°C, while the lowest variation was 2.4-fold in MHC IIA fibres at 15°C. Given that specific force is the ratio of fibre P_0 and CSA, the relationship between these two variables was plotted (Figure 2.3C and D). Each data point in the scatter diagrams represents the mean specific force reported from an individual publication. For reasons of clarity error bars are not shown. The standard errors of both P_0 and CSA were 3.8% of the mean. Significant correlations ($p< 0.01$) between CSA and P_0 were observed for both MHC I and IIA fibres in the 15°C group (black, filled symbols) but not the 12°C and >18°C groups. Therefore, only the regression line for the 15^0C group is displayed.

2.3.3 P_0 vs CSA relation following adjustments to data for fibre swelling, shape and SL.

As expected, removing the correction for the 20% swelling of fibre diameter which was applied by some, but not all studies, improved the correlation between P_0 and CSA, significantly so in the case of the 15°C group. Converting CSA measurements from CSA_A to represent the value which would have been obtained if CSA_E had been used by using $Q^{AE} = 0.9$, significantly increased the P_0 vs CSA correlation. No improvement in correlation was achieved for converting CSA_C to CSA_E. Correcting specific force values to represent the specific force which would have been measured at a SL of 2.75μm improved the P_0 vs CSA correlation slightly but not significantly for both MHC I and IIA fibres Table 2.2.

The extent the methodological adjustments made in the present investigation reduced the variation in published specific force data which can be seen by comparing the filled (after adjustments) and unfilled (before adjustments) box and whisker plots in Figure 2.3A and B with the open box (before adjustments). Furthermore, a tighter clustering of the data points in the 15°C group following adjustments can be seen for both MHC I and IIA fibres by comparing Figure 2.3E and F with Figure 2.3C and D. This mirrors the improved consistency in data for the 15°C group but not the other temperature groups, displayed numerically by the correlation coefficients for each temperature group in Table 2.2. The improvement in the coefficient correlation in the 15°C group indicates that ~30% of the variation was accounted for by the methodological factors considered.

2.3.4 Comparison of MHC I and IIA fibres

Figure 2.5A displays the mean difference between MHC IIA fibre and MHC I fibre specific force, as reported by the individual studies included in the present investigation. The data are scattered around a line expected for MHC IIA specific force being 19% greater than MHC I specific force, indicating that on average MHC IIA fibres are consistently found to be ~20% stronger than MHC I fibres, within studies. Figure 2.5 includes data for all temperatures, so includes data from groups (12°C and >18°C) in which there was very little correlation between P_0 and CSA, but nevertheless, the scatter around the mean line is modest. Furthermore, the Bland Altman plot in Figure 2.5B indicates that there was no significantly proportional bias ($p > 0.05$), showing that there is a good level of agreement in the mean difference between MHC I and IIA specific force.

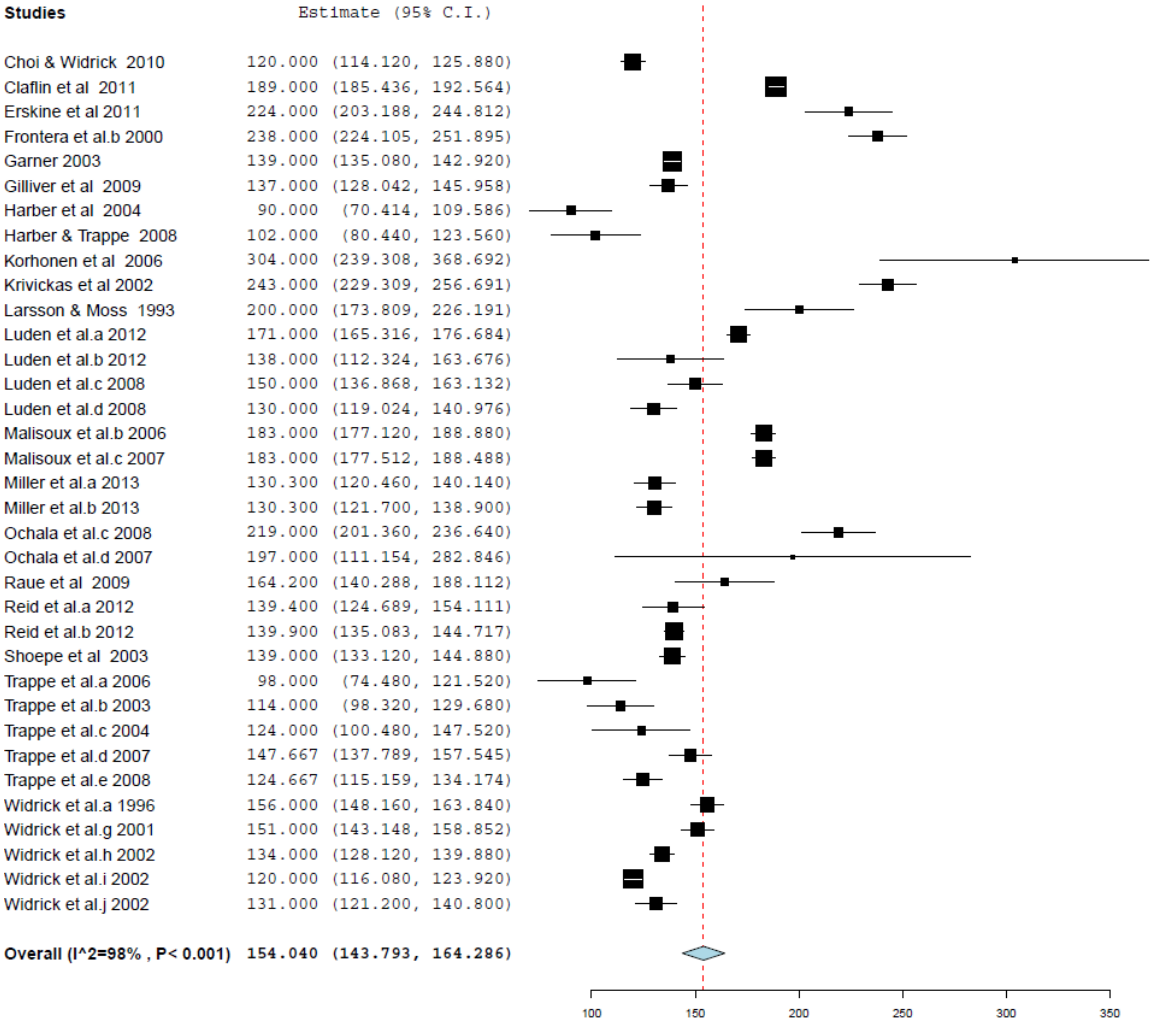


Figure 2.2. A Forest plot of the published specific force data measured from MHC IIA fibres from young humans at 15°C.

Up to 2.4-fold variability in specific force was observed in MHC IIA fibres at 15C, which was the lowest degree of variability observed among the 6 different groups of data sets compiled in the present systematic review. Nevertheless, the reported heterogeneity was extremely high, as indicated by the I^2 value from the Forest Plot output.

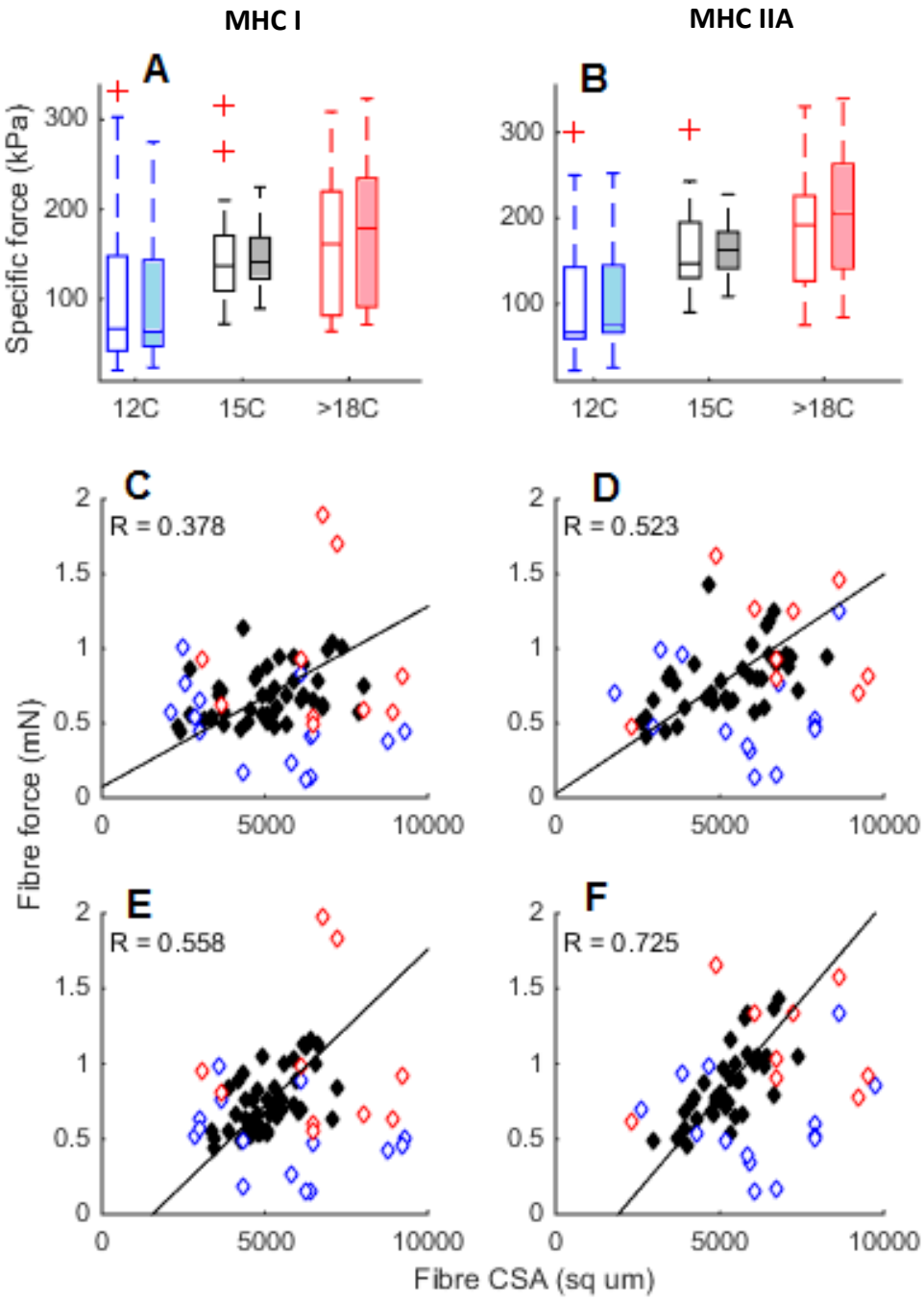


Figure 2.3. The variation in published, human MHC I and MHC IIA fibre specific force, before and after methodological corrections were applied.
A: MHC I fibres: box and whisker plots of published, mean specific force values for different temperature groups. See section 2.2.10 for the specifications of plotting the box and whiskers plots. The open boxes are the data as published, and the filled boxes are the data after the adjustments for methodological differences described in the section 2.2.3. B: the same as A, for MHC IIA fibres. C: MHC I fibre scatter diagram of P_0 against CSA. Each point is the mean result from a separate study. D: as C, for MCH IIA fibres. E: the data presented in C following methodological adjustments described in section 2.2.3. F: as D following methodological adjustments. Specific force data are from 12°C (blue), 15°C (black) or >18°C (red).

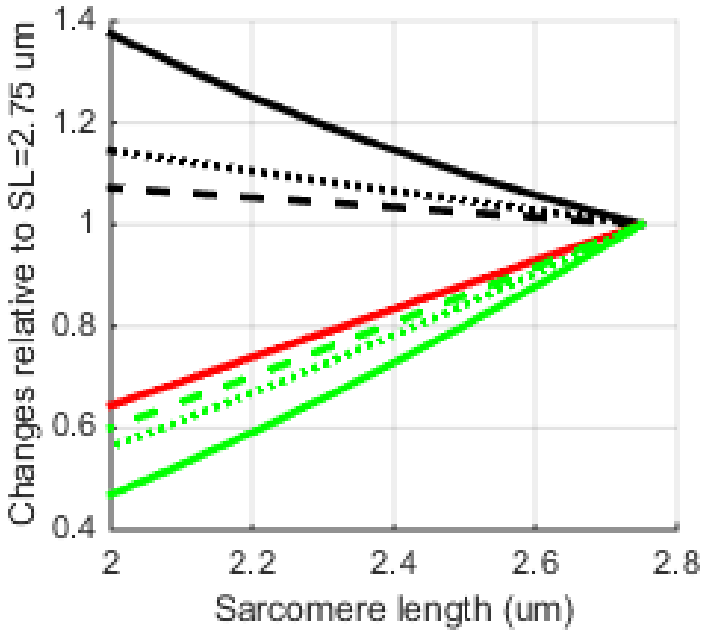


Figure 2.4. Changes with sarcomere length, in P_0 (red), CSA (black) and P_0/CSA (green).

All values are shown relative to values at SL of 2.75μm, the optimal length for force development in human skinned fibres (Gollapudi and Lin, 2009). Full lines show constant volume behavior, as observed in crayfish skinned fibres (April and Brandt, 1973). The dotted and dashed lines are from observations on frog mechanically fibres in chloride and propionate solution respectively (Matsubara and Elliott, 1972).

	MHC I			MHC IIA		
Temperature groups	12^{0}C	15^{0}C	>18^{0}C	12^{0}C	15^{0}C	>18^{0}C
Data as published	**0.459**	**0.378**	0.003	0.115	**0.523**	0.098
After removal of swelling correction	0.390	**0.510**	0.003	0.014	**0.666**	0.098
+ conversion of CSA-A to CSA-E	0.390	**0.523**	0.003	0.014	**0.705**	0.098
+ correction for sarcomere lengths	0.313	**0.558**	0.002	0.099	**0.725**	0.090
	Type 1 & Type 2A combined					
	12^{0}C	15^{0}C	>18^{0}C			
All above adjustments	0.116	**0.631**	0.028			
	All temperatures and both fibre types					
After all the above adjustments and also adjustment for temperature	**0.149** N=132					
Publication Groups	Group P	Group Q	Group R	Group S		
After all the above adjustments (N at 12, 15 & >18^{0}C)	**0.393** N=24 (18 0 6)	**0.651** N=47 (0 45 2)	**0.580** N=18 (0 13 5)	**0.439** N=23 (10 13 0)		

Table 2.2. The correlation coefficients of the mean P_0 and CSA values reported in different publications for MHC I and IIA fibres tested at 12C, 15C or >18C.
The correlation coefficients of each adjustment made in the present study, described in section 2.2.3. Values that are significantly greater than zero (pnull<0.05) are shown in bold.

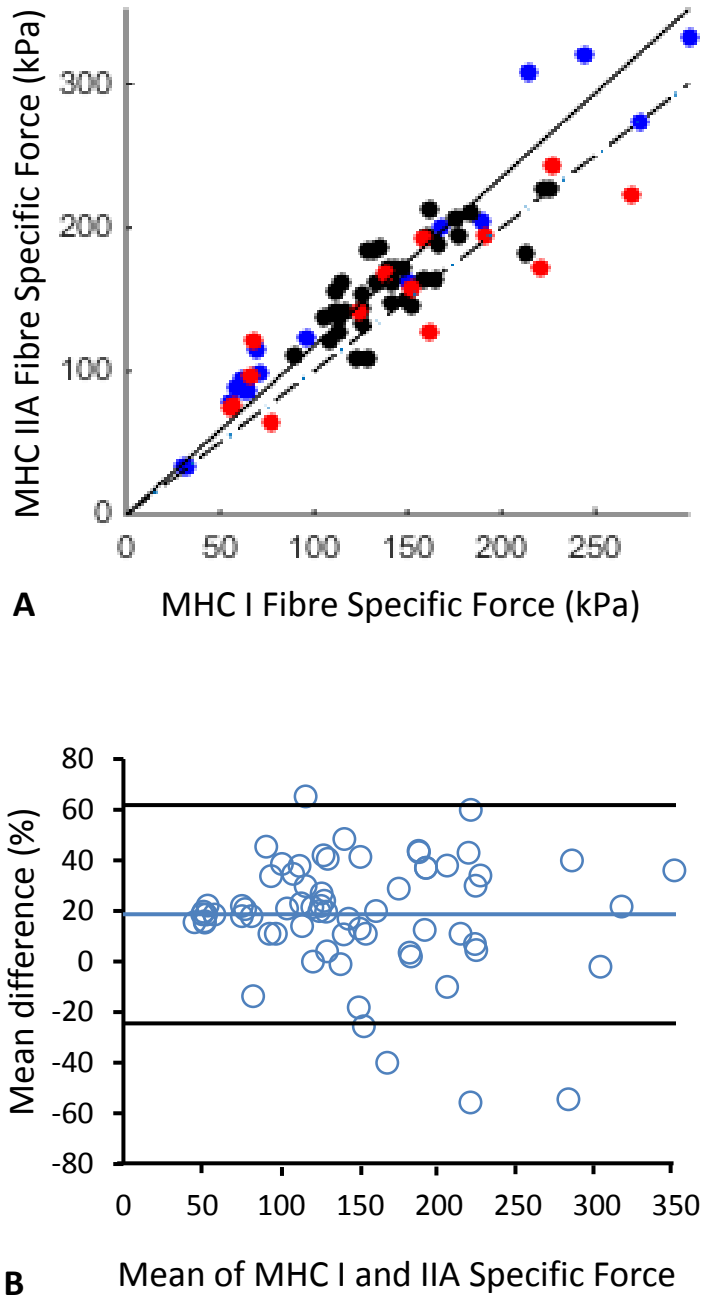


Figure 2.5. Comparison of specific force in MHC I and IIA fibres within studies included in the present systematic review.
A: Each point is the mean result from one publication, measured at 12°C (blue circles), 15°C (red circles) or >18°C (red circles). The dashed line is the line of identity (y=x) and the full line is y=1.174 * x. 1.174 is the geometric mean of the ratios of the 62 matched pairs of measurements. **B:** The Bland Altman plot indicates the mean difference between MHC I and IIA fibre specific force (19%, blue line), and upper (61.8%, upper black line) and lower (-24.4%, lower black line) confidence intervals. There was no significant ($p > 0.05$) proportional bias, indicating that there is a good level of agreement in the difference between MHC I and IIA fibre specific force.

2.3.5 Compilation of data from different fibre types and temperature groups

When specific force data from MHC I and MHC IIA fibres were combined, the correlation between P_0 and CSA was intermediate, between that reported for the two groups separately (Table 2.2). When the data from all three temperature groups were combined, accounting for all previous adjustments, the correlation between P_0 and CSA was dramatically reduced compared to that from the largest of the single temperature groups (15°C) (Table 2.2). However, when the data were separated based on research group the correlation within each of the four groups was restored to a considerably stronger level Figure 2.7.

The analyses were repeated, excluding data sets which were obtained from publications which studied cohorts over 40 years of age. Figure 2.8 demonstrates that the difference in results published between research groups is a valid conclusion even with the smaller sample of data sets.

The fact that a good correlation between P_0 and CSA exists within publications but not in the combined data implies that different specific forces are being reported by the different publication groups. An analysis of variance comparing these groups confirmed that there was essentially zero probability that all groups' reported specific force values (Figure 2.3) were from the same population Figure 2.8.

2.3.6 Differences in the activating solutions

Of the chemical constituents present in the activating solutions used by studies included in the present investigation, a correlation between PCr concentration in the activating solution and the reported specific force values was observed. This is illustrated in Figure 2.9, where results are grouped into 4 categories based on the concentration of PCr used. There are representatives of several publication groups in each of the PCr

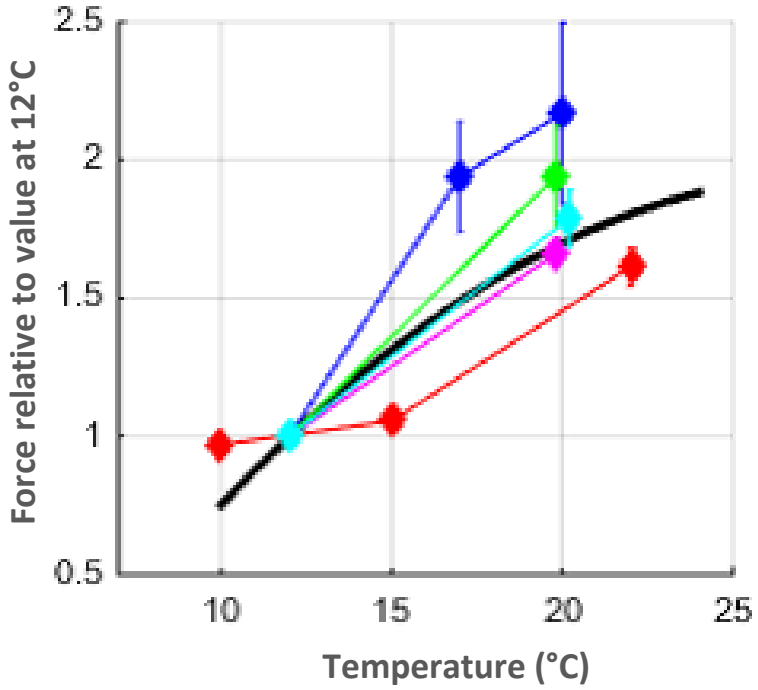


Figure 2.6. Temperature dependence of force in human muscle.
The black line is from *in-vivo* experiments (Ranatunga et al., 1987), and the coloured lines are observations in which individual skinned human fibres were observed at two or more temperatures.(blue: Bottinelli et al. (1996); red: Godt and Lindley (1982); green: MHC I from Stienen et al. (1996); pink : MHC IIA from Stienen et al. (1996); cyan: Brenner et al. (2012).

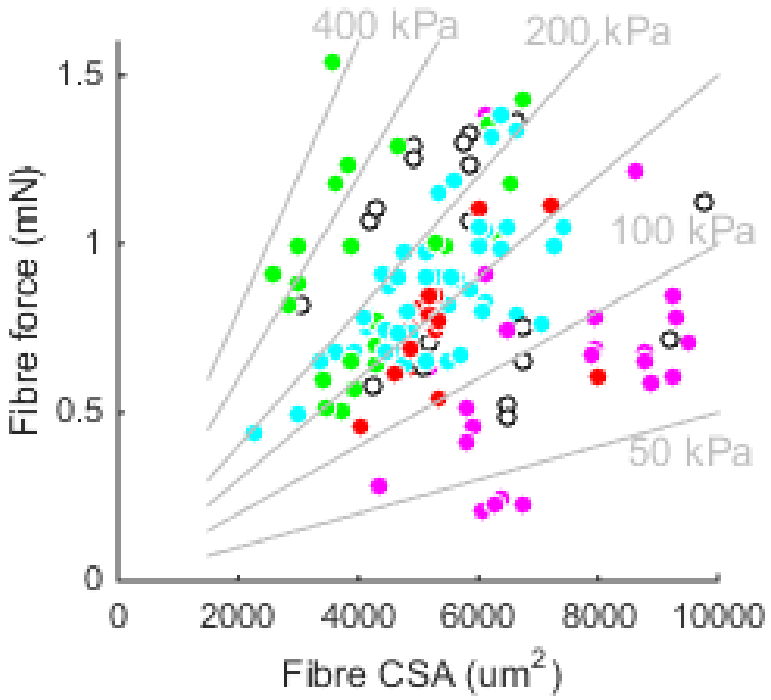


Figure 2.7. Relation of fibre force to fibre CSA after all adjustments described in the text.

Adjustments for methodological differences were made, MHC I and MHC IIA results were combined (by increasing the MHC I specific force values by a factor 1.17), and all data were adjusted to 15^{0}C as described in the text (sections 2.2.3 and 2.2.5). The different coloured markers refer to the different publication groups, as shown in Figure **2.1** are colour coded correspondingly. The grey lines indicate a range of specific force values.

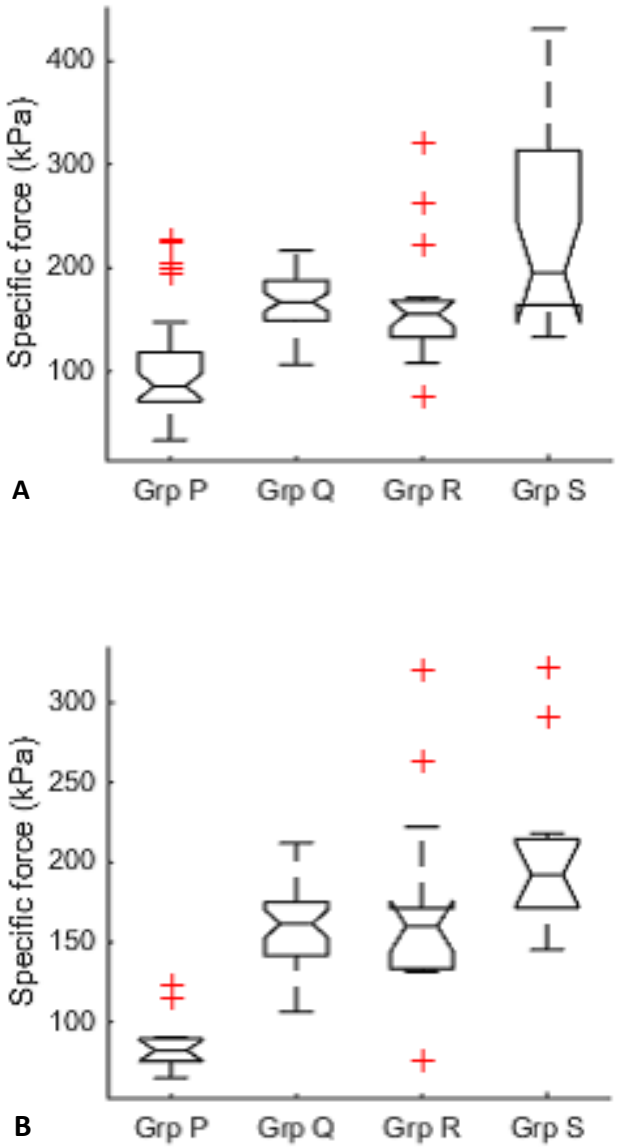


Figure 2.8. Comparison of MHC I and IIA fibre specific force by publication group. **A:** Box and whisker plots were plotted as described in section 2.2.10. A Kruskal-Wallis one way Anova gave a Pnull value of ~10^{-9} for the hypothesis that all specific force values are drawn from the same population. **B:** The same as A, but for a restricted subset of the sources as described in section 2.2.9, which caused Pnull to become ~ 10^{-7}.

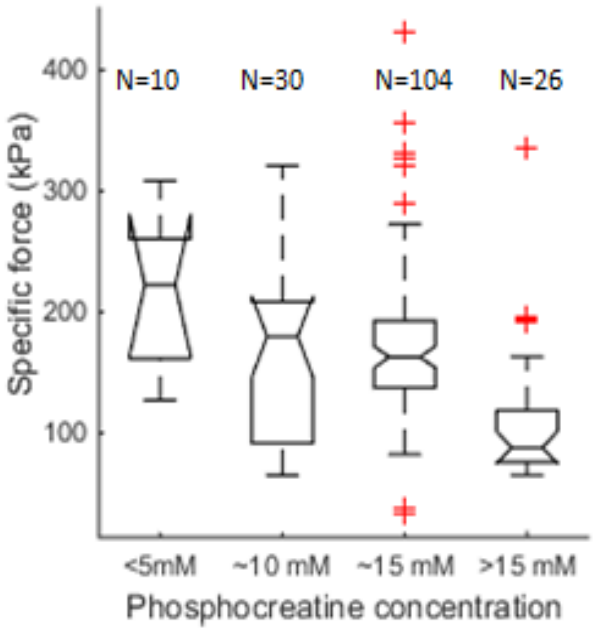


Figure 2.9. Comparison of MHC I and IIA fibre specific force in studies grouped by the Creatine Phosphate (CP) concentration in the activating solution.
In a few cases PEP was used instead of CP. The group boundaries have been chosen so that each group contains data from at least two of the publication groups (P through S). A Kruskal-Wallis one way Anova gives a Pnull value of about 10^{-5} for the hypothesis that all specific force values are drawn from the same population. The median value for the 15mM group is significantly different from all the others, but there are no significant differences between any of the other groups.

concentration groups. There is a significant trend for the median value of specific force to be less when the PCr concentration is greater.

2.3.7 Date of publication

No significant relationship was observed between the date of publication and the adjusted specific force reported. Dividing the sources into quartiles by year of publication showed no difference in either the mean or the standard deviation of specific force between these chronologic groups.

2.1 Discussion

A high degree of variability in specific force reported in the human skinned fibre literature was observed in the present investigation, for example, a greater than 8-fold difference was observed in MHC I fibres studied at 12°C. The variability was shown to occur between, but not within publications and, on a broader scale, between but not within research groups. Methodological factors were identified that reduced the variability, which suggests that relatively consistent data can be obtained using the skinned fibre technique, compared to the initial variability reported. The impact of unknown factors such as the true amount of swelling are also discussed.

2.1.1 Inclusion of 153 data sets based on 2 way ANOVA results

Specific force measured from skinned fibres of different muscles and from either males or females was not found to be significantly different ($p > 0.05$). These findings are supported by data from two studies (Miller et al., 2013, Reid et al., 2012) which report specific force measurements for both male and female groups of participants, observing no significant differences. In contrast, Yu et al. (2007) state that specific force from

skinned fibres was significantly higher in females compared with males. However, that skinned fibre specific force data from males and females was grouped together in fourteen publications for both MHC I and MHC IIA fibres suggests that they are not significantly different. Furthermore, reliable specific force measurements from whole muscle show no significant difference between males and females (O'Brien et al., 2010). Therefore, analysis was carried out in the present investigation assuming that male and female skinned fibre specific force data are not significantly different. With regard to the muscle studied, the vast majority (64%) of publications measured specific force from Vastus Lateralis fibres with a minority using soleus (15%), gastrocnemius (12%) or another muscle (<10%). Based on the two-way ANOVA results, data sets obtained from different muscles were combined for analysis in the present investigation.

2.1.2 Specific force variability

A large heterogeneity (Figure 2.2) and variation in specific force values reported in the literature for human skinned skeletal muscle fibres was demonstrated (Figure 2.3). The high degree of variability in published MHC I and IIA fibre specific force is highly unlikely to be due to differences in physiological specific force generating capacity in the human muscles that were biopsied. Clearly, factors in the preparation, in the measurement and in the data analysis have caused the results to vary across such a wide range.

2.1.3 Effect of standardising methodological factors on specific force variability

Eliminating the influence of three methodological differences between studies reduced the large variability observed in specific force values reported in the literature Figure 2.3. This finding supports that differences in specific force measurements between studies are not due to differences in participants from control groups or their muscle

biopsy samples, but are due to methodological differences in how skinned fibres are treated by different publication groups. If the variance had been unaffected by accounting for methodological differences, the implication would have been that using a similar method produces inconsistent results, questioning the validity of the skinned fibre technique.

Removing the correction for fibre swelling known to occur, unifying the assumption made regarding fibre shape among all included studies, and adjusting reported P_0 based on the SL-force relationship significantly increased the correlation between P_0 and CSA. The improved P_0 vs CSA correlation coefficients following data adjustments indicate that ~30% of the variation in reported specific force values is explained by the methodological differences between studies accounted for in the present investigation. Hence, the findings of the present study support that more consistent data can be obtained using the skinned fibre technique than could originally be discerned from the large variation which exists in published specific force values. Furthermore, this shows that there are additional, and major reasons for the dispersion of data other than the differences in technique and data processing which have been considered so far.

No improvement in P_0 vs CSA correlation was observed when fibre shape assumed was converted from CSA_C to CSA_E, potentially because the 'width' was not the major axis, as is assumed in the present investigation. The P_0 vs CSA correlation was stronger for MHC IIA fibres than MHC I fibres in the data as originally reported and after adjustments for methodological factors. This may be due to the fact that there is an unknown amount of error in the CSA measurements (discussed later), so the higher P_0 produced by type IIA fibres reduces the impact of any error in CSA measurement on the P_0 vs CSA relationship.

O'Brien et al (2010) measured specific force *in vivo* and accounted for all confounding methodological factors, yielding reliable measurements. A high correlation of 0.98 is observed between the mean P_0 and CSA value from whole quadriceps muscle across the experimental groups. The significantly improved P_0 vs CSA relation following adjustments for methodological differences between studies is closer to that observed *in vivo*. This implies that the P_0 vs CSA relation of skinned fibres is more representative of that *in vivo*, than could be discerned when visually inspecting the published specific force data as originally reported (Figure 2.3A and B). This suggests that the skinned fibre technique can be used to study the contractile properties of skeletal muscle, however, unknown factors which contribute to the variation of specific force data between studies persist.

Quadriceps muscle specific force measured in different groups of humans *in vivo* has been shown to be ~565 kPa (O'Brien et al., 2010). The average specific force measurement taken from MHC IIA fibres at 15°C was 154 kPa. Given the temperature sensitivity of skinned fibre specific force, even adjusting this value to that which would be obtained at a physiological temperature would yield a lower specific force than that observed *in vivo*. Therefore, while the present study has shown that the skinned fibre technique is more representative of *in vivo* specific force production than could initially be understood there is clearly a need to optimise this technique.

2.1.4 Comparison of MHC I and MHC IIA fibres

Figure 2.5 displays MHC IIA fibre specific force against MHC I fibre specific force measured by the same individual study. The fact that the scatter of data points around the mean line is modest indicates that the factors causing the variation in the P_0 vs CSA relationship are not prevalent *within* the individual studies, but *between* individual

publications. This implies that specific force results from individual studies are valid, something which has not previously been demonstrated, and could not be understood from the huge variation observed in specific force measurements reported in the literature (Figure 2.3). Therefore, conclusions drawn from specific force measurements in a given study may represent the effects of a given intervention or physiological condition while comparison of results between studies remains difficult. That variation exists between, but not within studies supports that differences in specific force measurements are largely due to methodological differences between studies.

That MHC IIA fibres are intrinsically stronger than MHC I fibres is well accepted (Schiaffino and Reggiani, 2011). This is supported by an analysis demonstrating that investigations which study a higher *n* number of fibres are more likely to observe that MHC IIA fibres have a greater specific force than MHC I fibres (Miller et al., 2015). The present investigation shows that MHC IIA fibres appear to consistently be 20% stronger than MHC I fibres on average, across 62 publications Figure 2.5.

2.1.5 Adjustment for temperature differences

Data from different temperature groups which had been adjusted for methodological factors (fibre swelling, shape, SL and type) were combined by converting specific force measured at temperatures other than 15°C to the expected value at 15°C. The combined specific force data exhibited a greatly reduced P_0 vs CSA correlation compared with that from the 15°C group. A possible reason is that the combined data contain more publication groups that the 15°C group, since each temperature sub-group does not contain the same number of publication groups.

When the combined specific force data were separated by publication group (Figure 2.7) the correlation within each of the 4 groups was restored to a much stronger level (Table 2.2), even though most of the groups contained data from more than a single temperature. Therefore, the variability in specific force data does not only occur between but not within individual publications, but occurs on a broader scale *between* but not *within* publication groups. This indicates that methodological practices of research groups as a whole and not just individual publications, influence the variation in specific force measurements observed in the present investigation. The finding that within research groups there is a reasonable concordance between studies is in agreement with the result of Krivickas et al (2011) who combined individual fibre results from six studies made in the same laboratory to obtain a large and consistent data set.

2.1.6 Swelling of skinned fibres

When skeletal muscle fibre segments are mechanically skinned or chemically permeabilised, they swell (Matsubara and Elliott, 1972). Fibre swelling upon skinning is thought to be an osmotic phenomenon caused by the removal of soluble proteins from the environment of the myofibrils. The exclusion of these proteins from the filament lattice draws water out from it. The swelling after permeabilisation can be reversed by providing a similar osmolar environment to that before skinning, for example by using dextran (Andrews et al., 1991, Godt and Maughan, 1977). The publications included in the present investigation commonly refer to skinned fibre swelling as "the 20% swelling that is known to occur during skinning," although the amount of swelling measured has been found to vary. Therefore a possible reason for the large variation in specific force is that skeletal muscle fibres swell by a variable amount when skinned.

The two references most commonly cited to support the fact that skinned fibre diameter swells by 20% following skinning do not provide strong evidence to support this statement. Moss (1979) studied frog muscle fibres at 5°C and does contain a single sentence in the method section that the fibre diameter 'increases by about 20%' upon skinning. However, no data is presented from which the range of the diameter swelling can be determined, and the number of observations is not specified. Godt and Maughan (1977) studied frog fibres at lower temperatures and note the amount of swelling observed. The mean increase in CSA is 2.32 fold (± 0.54 SD), which corresponds to an approximate diameter increase of 52% (± 18% SD), much higher than the 20% this work is cited for. This suggests that reference is being systematically misquoted and skinned fibre CSA may be being adjusted by an incorrect amount. Furthermore, the fact that Godt and Maughan (1977) are cited as the source first observing that skinned fibres swell by 20% is surprising, since they explicitly state that the swelling observed in their study was greater than that observed by other investigators: *"…We observed much more swelling than did other workers."*

The significance of knowing precisely how much swelling occurs is evidenced by Blauuw et al (2009) who quantified the amount of swelling of skinned fibres by comparing the CSA of skinned mouse fibres to the CSA of fibres in histological section. An 11% increase in fibre CSA due to skinning related swelling was observed in their experimental group compared with a 25% increase in fibre CSA in their control group. Such a variable amount of swelling has serious implications for the interpretation of the effect of any intervention or condition on muscle quality, as indicated by specific force measurements. Furthermore, the fact that the amount of swelling is likely to be variable in different skinned fibres shows that correcting specific force by a constant factor is inappropriate. However, these swelling measurements should be interpreted with caution

since the amount of swelling was calculated by comparing skinned fibre CSA to that of fibres in histological sections where an unknown amount of CSA shrinkage occurs.

The reasons for the large variability in the swelling are unknown, although Matsubara and Elliott (1972) observed different amounts of swelling at different sarcomere lengths. If similar variations in swelling occur in mammalian fibres and if slight differences in technique in different labs influence the amount of swelling then it would be a likely cause of the differences reported in specific force. This suggests that an investigation into the magnitude and variability of skinned fibre swelling should be conducted. Alternatively, another method could be used as an index of fibre size, which is itself a proxy measurement for the number of crossbridge sites in parallel with one sarcomere.

2.1.7 Other methodological factors potentially contributing to the inter-research variability

The single fibre skinning procedure often includes treatment with detergent, usually either Triton X100 or Brij 58. Triton was used in 16 of the 17 studies from publication group P and by only 4 other investigations across the other publication groups. The adjusted specific force was significantly lower in studies which used Triton (121 ± 8, n = 50) compared to those which used Brij-58 (186 ± 6, n = 201). However, the effect of Triton could not be isolated and attributed to the reduced reported specific force using systematic review methodology, and experimental work would be required to test this hypothesis. Indeed, the lower specific forces reported by publications which used Triton could be due to other methodological factors causing lower specific force values to be reported by a given publication group. The same principle applies to investigating the

effects of different skinning solutions used by different publications, which are distinguished by the anion used in solution, either Chloride or Proprionate.

In the present systematic review, four publications included immediately froze their biopsy samples in liquid Nitrogen, or isopentane cooled in liquid Nitrogen. In one study, from Group P, the adjusted specific forces were very low (below 75 kPa), although forces from two other studies from Group S were above 150 kPa. However, two comparative studies compared the effects of immediate freezing with immersion of the muscle biopsy in cold skinning solution and observed that freezing reduced skinned fibre force by ~60-70% (Frontera and Larsson, 1997, Larsson and Moss, 1993). Therefore, immediate freezing seems to reduce skinned fibre force development.

2.1.8 Differences in the activating solutions

A trend for the median value of specific force to be lower when the CP concentration was greater, was observed when data were grouped into four categories based on the concentration of CP used (Figure 2.9). This result must be interpreted with caution, since the papers using a particular CP concentration are likely to share other technical details which are unreported. Nevertheless, a possible mechanism of high CP concentrations which might cause lower specific force, considered below, is that higher contaminating concentrations of inorganic phosphate (P_i) may be generated by higher CP concentrations, reducing specific force.

A linear relationship between skinned fibre force and the log of Pi concentration at concentrations above 0.1mM has been reported in rabbit muscle (Karatzaferi et al., 2004). Based on this relationship, the P_i contamination in the activating solutions used by Garner and Widrick (2003) and Pathare et al. (2005) can be calculated from their

observations of force to Pi concentration, which are 0.25 and 0.55mM respectively. Both publications used activating solutions containing ~20mM CP and reported specific force values less than 150kPa. Assuming a reduction in the CP concentration to ~10mM would reduce the Pi contamination by 50%, an approximate 10% increase in specific force would be expected, which is too small an effect to explain the trend observed in Figure 2.9. Therefore, hypothesis that contaminating P_i which lowers specific force is generated as a result of higher CP concentrations is rejected.

Using systematic review methodology the impact of individual chemical constituents of a given activating solution could not be assessed. Factors such as the salt used to adjust ionic strength or the pH buffer may also affect the skinned fibre contractile response but would need to be tested experimentally, as will be discussed in Chapter 4.

2.1.9 Summary and Conclusion

The aim of the present investigation was to assess the variability in published specific force values from human, skinned skeletal muscle fibres, in order to better understand the reliability of data obtained using this technique. Greater than 8-fold variability was observed in reported specific force values for a given fibre type tested at the same experimental temperature, suggesting that the skinned fibre technique does not yield reproducible data across different labs. However, the MHC I:MHC IIA specific force ratio was consistent within individual studies, demonstrating that variability occurs *between* but not *within* publications. This trend was shown to occur on a broader scale, with consistent specific force results obtained *within* but not *between* research groups (Figure 2.7). The consistency within individual studies and research groups suggests that reported results are reliable. This important finding was not previously known and is not

conveyed by the large and previously unexplained variability in published specific force data.

Accounting for methodological differences between studies reduced the variance in specific force reported, supporting the hypothesis that the large variation was not due to physiological differences between control groups from different studies. Furthermore, the P_0 vs CSA relationship (at 15°C) was significantly improved when methodological differences were accounted for, indicating that ~30%, but not all of the variation was due to the factors considered. The improved P_0 vs CSA relation also demonstrates that the skinned fibre technique is considerably more reflective of the relation between muscle P_0 and CSA *in vivo*, than the large variation in published data suggests.

3 General Methods

The experiments conducted in the present thesis were carried out on human skinned fibres. The steps taken to obtain and prepare a biopsy sample for measurements of individual skinned fibre specific force will be described in the following Methods section. The procedures used for identification of skinned fibre MHC isoform expression and quantification of myosin content will also be presented.

3.1 Ethical approval

The experimental work in the present thesis was conducted on Vastus Lateralis skeletal muscle samples from participants who were either, 1) Young, healthy controls, 2) Frail hip fracture patients, or 3) Highly physically active elderly individuals. Ethical approval was granted by the Fulham Research Ethics Committee in London, to obtain muscle samples from young, healthy individuals and from physically frail hip fracture patients (Ethics ID: 12/LO/0250). Muscle biopsy samples were obtained from highly physically active elderly individuals as part of a different investigation (Pollock et al., 2015), previously approved by the Wandsworth Research Ethics Committee in London (Ethics ID: 12/LO/0457). All procedures ran in accordance with the declaration of Helsinki (1964).

Professor Stephen Harridge was responsible for taking muscle biopsy samples from young, healthy participants and highly physically active, elderly individuals. Participants visited the Centre of Human and Aerospace Physiological Sciences at King's College London to undergo this procedure. Muscle samples from hip fracture patients were collected by registrars working with Mr Marc George at St Thomas' Hospital in London. The different muscle sampling procedures which were utilised are outlined in section 3.3.

3.2 Participant inclusion and exclusion criteria

Participants from all experimental groups were fully informed of any associated risks and provided informed consent. Healthy, young participants who were between the ages of 18-40 completed a health questionnaire (Appendices section 8.1). Grounds for exclusion, included if they were taking any medication, had any musculoskeletal, cardiovascular or neurological problems. The participants who volunteered their muscle sample for a given study are outlined where relevant in each chapter of this thesis.

3.3 Skeletal muscle tissue sampling

The muscle biopsy samples for the young, healthy participants and physically active, elderly participants were obtained using the same biopsy procedure. A portion of the mid-thigh of the participants' preferred leg was shaved and the skin was cleaned using chlorhexidine gluconate. Two percent lidocaine was used as an anaesthetic prior to a 5mm incision in the skin overlying the biopsy site, made with a scalpel. A Bergstrom biopsy needle was inserted through the incision and a biopsy was taken using suction, yielding a sample of approximately 200mg in weight. Approximately 60mg of the biopsy sample was prepared for experiments examining the contractile properties of single, skinned fibre segments.

Muscle tissue samples were obtained from hip fracture patients who were scheduled to undergo dynamic hip screw insertion surgery. During the routine exposure of the hip during surgery, a portion of the Vastus Lateralis muscle inevitably becomes detached. Rather than discarding this muscle sample, the surgeons placed the sample into a relaxing solution which had been cooled on ice. The muscle sample was collected immediately following surgery and prepared for skinned fibre experiments. The number

of biopsies used for the studies described in the present thesis is specified in each experimental chapter.

3.4 Membrane permeabilisation of fibre bundles

The portion of the muscle biopsy sample to be used for skinned fibre experiments was immediately placed into a petri dish containing relaxing solution (Table 3.1), which had been cooled on ice to approximately 4°C. The muscle sample was then dissected into bundles of muscle fibres under a stereo microscope (Zeiss, Stemi 2000-C) with a separate light source (Zeiss SteREO CL 1500 ECO). Custom-made dissection needles were used to separate a bundle of approximately 50-100 fibres from one end of the tissue sample, before gently pulling the bundle off from the rest of the sample using forceps, ensuring not to overstretch the fibres. The fibre bundles were tied at each end to glass capillary tubes using surgical silk (LOOK SP102) and were extended to approximately 110% of their slack length.

Still attached to capillary tubes, the fibre bundles were placed into tubes containing relaxing solution with 50% v/v glycerol (Table 3.1), in order for the sarcolemma to be chemically skinned at 4^0C for 24 hours. Following skinning of the sarcolemma, the bundles were transferred into a -20°C freezer while still in skinning solution and stored for up to three weeks until they were processed for long term storage using a sucrose cryo-protectant.

3.5 Long-term storage of fibre bundles

Bundles were incubated in standard relaxing solutions containing concentrations of 0.5, 1, 1.5 or 2M sucrose (Table 3.1), in order of increasing concentration, for thirty

minutes in each solution. Immediately following incubation, bundles were snap frozen in isopentane (GPR - RECTAPUR) cooled to -80^{0}C using dry ice and subsequently stored at -80°C in cryo-viles . On the day of an experiment a bundle would be de-sucrosed by incubating it for 30 minutes in each sucrose solution in reverse order (2M → 0.5M) before placing the bundle in relaxing solution. All skinned fibres studied in the present thesis underwent sucrose treatment for long term storage in order to standardise the treatment of the fibres prior to experimentation.

3.6 Preparation of skinned fibres for mechanical experiments

Following the de-sucrosing of a fibre bundle, fine forceps were used to dissect 1-2mm permeabilised fibre segments from the bundle under a stereo microscope with a separate light source. Individual fibres were dissected when a bundle was in relaxing solution cooled on ice to approximately 4°C. Aluminium t-clips (Photofabrication Ltd, Cambridgeshire, England) were attached to both ends of each of the fibres using custom-made dissection needles, before a single fibre was transferred to relaxing solution (pCa 9.0) in an experimental chamber. Each t-clip was connected to a hook, one of which was attached to a force transducer (Kronex, AE-801), thus suspending the fibre in solution between two hooks. The main components of the experimental apparatus used for mechanical experiments are described in section 3.7.

Solution	ATP (mM)	EGTA (mM)	$MgCl_2$ (mM)	Imidazole (mM)	KCl (mM)
Relaxing	4.05	2	2	10	100
Skinning	Same as relaxing solution but with 50% v/v glycerol.				
Sucrose	Same as relaxing solution but with either 0.5M, 1M, 1.5M or 2M of sucrose in solution.				

Table 3.1.The chemical constituents of solutions used to prepare a muscle biopsy sample for mechanical experiments.
The chemical compositions of the relaxing solution used to prepare bundles of skinned fibres for chemical skinning, the skinning solution, and the sucrose solution used as a cryoprotectant to process bundles of skinned fibres for long term storage at -80°C are detailed.

3.7 Permeabilised fibre test system

Measurements of the contractile properties of skinned fibres were carried out using a custom-made skinned fibre test system, which was set up, and a working protocol to collect skinned fibre specific force measurements was developed. As previously described, once prepared for an experiment a single fibre was suspended between two hooks in an experimental chamber containing relaxing (pCa 9.0) solution. The solution changing system consisted of six separate, clear plastic bottom chambers embedded in a custom-made, temperature regulated metal trough (NMR, Mill Hill, London, England) (Figure 3.1). The transparent floor of the metal trough was connected using waterproof silicone sealant to prevent the movement of experimental solutions between the different chambers. Furthermore, hydrophobic ink was applied to the grooves in which the hooks suspending the fibre were positioned, using a custom-made miniature paint brush. This prevented the solution in a given chamber from leaking out through these grooves, which would alter the amount of solution surrounding the fibre.

A light microscope (Olympus, BXFM-F) with both a x10 (BECK, x10/0.17) and x40 (Carl Zeiss, 40/0.75W 160/-) objective lens was connected to the base of the skinned fibre test system and was used to view the skinned fibre from above. A small mirror was used to deflect light from an external light source (SCHOTT, KL1500 LCD) through the bottom of the transparent floor of the metal trough and into a given microscope lens. The x10 lens was used for measuring fibre length while the x40 lens was immersed in the solution surrounding the fibre and used for measuring the diameter and depth of individual fibres, as well as for adjusting the sarcomere length. Measurement of skinned fibre sarcomere length and dimensions are described in section 3.8.

In order to transfer a skinned fibre from solution to solution during an experiment the metal trough could be manually moved up, down and sideways in either direction.

Hence the trough was moved to surround the fibre with a specific experimental solution contained in a given chamber. The upwards and downwards movement of the trough was activated by an air pressure system. The sideways movement was controlled by a system based on ball bearings which allowed movement of the trough over a fixed distance, corresponding with the distance between the different chambers. The temperature of each solution a skinned fibre was placed in was maintained at 15°C by a water cooler (Neslab RTE-300) which was connected to the side of the trough with silicone tubing, in order for water to be circulated around the hollow trough interior. The solution temperature was monitored during each experiment using a thermocouple (HANNA, HI93530).

In order to cause a skinned fibre to contract, the fibre was immersed in an activating solution (pCa 4.5) (Table 3.2) and the contraction was recorded using a force transducer which was connected to a signal amplifier (NMR, Mill Hill, London, England; Model EE2588). The amplifier was in turn connected to a data acquisition device (National Instruments, USB 6008) to convert the analogue (voltage) signal to a digital signal which was visualised in real time as a skinned fibre force trace using a custom written LabView programme (National Instruments, Newbury, England). Measurement of skinned fibre contractile properties is described in section 3.9.

3.8 Measuring sarcomere length and skinned fibre dimensions

Once mounted onto the skinned fibre test system, the overall fibre length was adjusted until an optimal sarcomere length (SL) of 2.75µm was obtained. The SL was cross referenced using two separate methods: 1) An eyepiece graticule which had been calibrated using a stage micrometer (Graticules LTD, Tonbridge, Kent) was used to measure adjustments to the SL when muscle fibre striations were plainly visible at x40 magnification in solution. 2) A video sarcomere length (VSL) camera (900B, Aurora

Scientific) mounted to the microscope of the skinned fibre test system was used to measure the SL at three points along the fibre at x40 magnification. This was to ensure that the SL was uniform along the whole length of the fibre. Once the SL measurements between the two methods were in agreement and the skinned fibre SL was optimal, the morphometric measurements of the fibre were taken.

The diameter of the fibre was measured in solution at x40 magnification using the VSL camera, at three points along the fibre, as is routine practice in the skinned fibre literature. The mean of the three diameter measurements was used to estimate the fibre cross-sectional area (CSA) assuming a cylindrical fibre shape. No correction was applied to the fibre CSA for the swelling which occurs during the skinning process, since the exact amount of swelling in each fibre is not known (discussed in section 2.1.6). The dial used to move the microscope objective up and down indicated the vertical distance moved in μm, with one full rotation of the dial in either direction equal to a movement of 200 μm. Fibre depth could therefore be measured by recording the vertical displacement of the x40 microscope objective, which was used to focus on the top and bottom of the skinned fibre. Fibre length was classified as the portion of the fibre between the t-clips and was measured at x10 magnification using the eyepiece graticule.

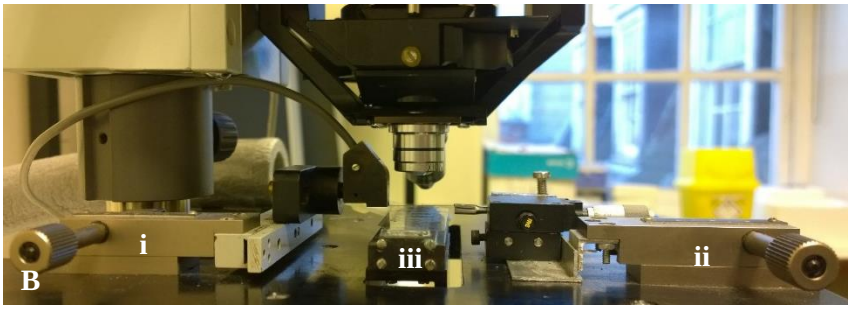
i - The mounting microscope, used to place an individual fibre into the set up.

ii – The thermocouple used to monitor experimental temperature.

iii – the external light source used to pass light through the bath trough into the microscope lens in order to view a given fibre.

iv – the skinned fibre test system where a given fibre was mounted.

v – the cooler which maintained a constant experimental temperature.



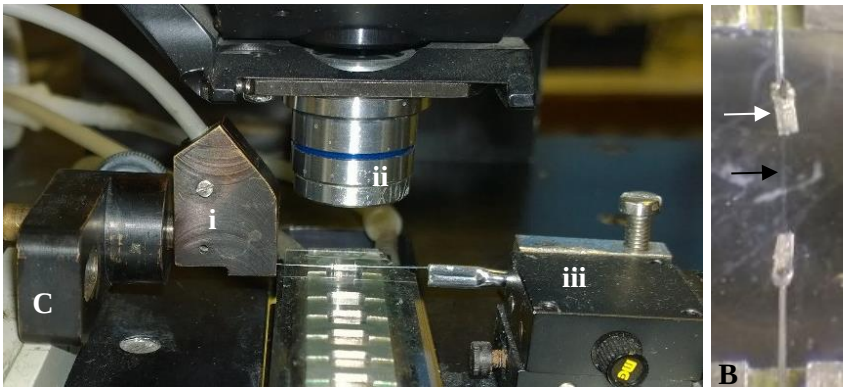


Figure 3.1. The skinned fibre test system.

A: The whole set up. The individual components are labelled and described in the text box.
B: The skinned fibre test system the fibre was mounted to. The micromanipulators used to adjust the position of the force transducer (i) or hook (ii) on either side of the bath trough which contains the experimental solutions (iii).
C: A zoomed in image of the skinned fibre test system. The force transducer hook (i) and stationary hook (iii) are pictured with the x10 microscope objective (ii) above the two hooks.
D: A single fibre (black arrow) suspended between the two hooks using t-clips (white arrow).

3.9 Measurements of contractile properties

Fibres were transferred from a relaxing (pCa 9.0) to a pre-activating solution where they were incubated for one minute before being transferred to an activating solution (pCa 4.5). The pre-activating solution had a lower Ca^{2+} and EGTA concentration than the activating solution, the reason for which is discussed in section 4.1. The chemical compositions of the two primary activating solutions used in the present experimental thesis are detailed in Table 3.2. As will be described in chapter 4, modified versions of the two primary activating solutions were made for experimental purposes, so their chemical composition is detailed in Table 4.1.

Force produced during a muscle contraction was recorded until a plateau in force production was reached. Peak isometric force (P_0) was classified as the baseline force signal of the fibre in the activating solution subtracted from the maximum force signal recorded. The P_0 was measured and time to half peak isometric force (t_{50}) was calculated using custom written Matlab software, the principles of which are described in section 0. Specific force was calculated as the P_0 normalised to the cross-sectional area (CSA) of the fibre. The time to 50% P_0 (t_{50}) was used as an indirect, approximate indication of the rate of force development. In order to obtain a direct measurement, reflective of the rate of cross-bridge cycling during a contraction, a slacken-restretch manoeuvre would need to have been carried out. This type of experiment was not conducted in the present thesis.

Activating solution A	Concentration (mM)	Activating solution B	Concentration (mM)
Imidazole	20	TES	60
Glutathione	0	Glutathione	10
KCl	62.7	K-propionate	20
$CaCl_2$	7	CaEGTA	24.8
EGTA	7	EGTA	0.17
CrP	14.5	CrP	20
ATP	4.7	ATP	4.65
pH	7.1	pH	7.1
Ionic Strength	180	Ionic Strength	200

Table 3.2. The concentrations of the chemical constituents comprising the two primary activating solutions used in the present PhD thesis to elicit isometric contractions from skinned skeletal muscle fibres.
The primary differences are the use of different pH buffers (Solution A: Imidazole; Solution B: TES), the use of Glutathione in solution B but not in solution A, and the use of a different compound in each solution to adjust the ionic strength (Solution A: KCl; Solution B: Potassium Propionate). Solution A is widely used by research groups which have published measurements of specific force from human skinned skeletal muscle fibres (Larsson and Moss, 1993, Trappe et al., 2003). Solution B is used by a research group which routinely measures specific force from non-human mammalian skeletal muscle (West et al., 2013).

3.10 Measurement of the baseline in activating solution

The movement of the metal trough when transferring a skinned fibre from one experimental solution to another, caused a brief, 300ms fluctuation in the force trace signal upon entry of the fibre into a new experimental solution. The fluctuation in signal occurred when a skinned fibre entered any new experimental solution, irrespective of whether or not the fibre was contracting. Therefore the 300ms signal fluctuation was classified as an artefact, which masked the baseline force signal of the skinned fibre response at the start of a muscle contraction. In order to accurately determine the 'true' zero point from which to calculate P_0 a custom written Matlab programme was developed by Professor Roger C. Woledge, the principles of which are described below.

On a given force trace, the point at which the fibre entered the activating solution was selected and classified as the start point of a fibre contraction (Figure 3.2). The P_0 was then selected to give the Matlab programme two reference points for the start and end of the contraction. As the signal artefact lasted for approximately 300ms, the first 300 data points, each corresponding to 1ms, were excluded from data analysis. From 300ms onwards the point at which 10% of P_0 occurred was calculated, from which point a straight line was extrapolated along the slope of the force trace. The reason for extrapolating backwards from 10% of the P_0 signal along the slope of a given force trace, was to account for the steepness of the force trace, which differed between different contracting skinned fibres. The point at which the line intersected the x-axis coordinate of the start of the contraction was considered to be the baseline force signal (Figure 3.2B) However, since the baseline force signal had been calculated, the point at which 10% P_0 occurred was subsequently altered. Therefore, 10% P_0 was re-calculated and the line to the start point of a fibre contraction was re-plotted repeatedly, until there was an almost 0% difference between the calculated start points. Once the zero point had been identified,

the Matlab programme calculated the P_0 and t_{50} for each contraction of an individual skinned fibre (Figure 3.2C)

The impact of removing the 300ms signal artefact from calculations of skinned fibre P_0 can be determined using the force trace illustrated in Figure 3.2. Here, the P_0 calculated using the Matlab programme, after removal of the 300ms artefact and calculation of the baseline force signal, was 0.56mN. The corresponding skinned fibre CSA, without correction for swelling, was 4727μm^2. The P_0 normalised to CSA gives a specific force value of 118kPa. However, if the extra 0.09mN of force signal which was excluded as signal artefact had been included in the P_0 calculation, the subsequent specific force for the same fibre would have been 16% higher, at 138kPa. Furthermore, the 0.09mN difference in P_0, due to the excluded signal artefact, would have an even greater effect on specific force calculations from fibres of smaller CSA. Therefore, the removal of the signal artefact, which was consistently found to be 300ms in duration, was used to eliminate the effect of signal artefact on the measurement of force.

3.11 Identification of muscle fibre types

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) was used to identify the fibre type of individual skinned fibres based on their myosin heavy chain isoform expression. The SDS PAGE protocol (section 8.2) needed to be optimised, as differentiation between MHC I and MHC II fibres was possible, but not initially between MHC IIA and IIX fibres. Therefore, a process of optimisation was undertaken to be able to differentiate between MHC I, IIA and IIX human skeletal muscle fibres in the current laboratory. In order to identify the three MHC isoforms expressed in human skeletal muscle, a sample containing all three isoforms was required. Several potential control samples were prepared for SDS PAGE by homogenising human skeletal muscle

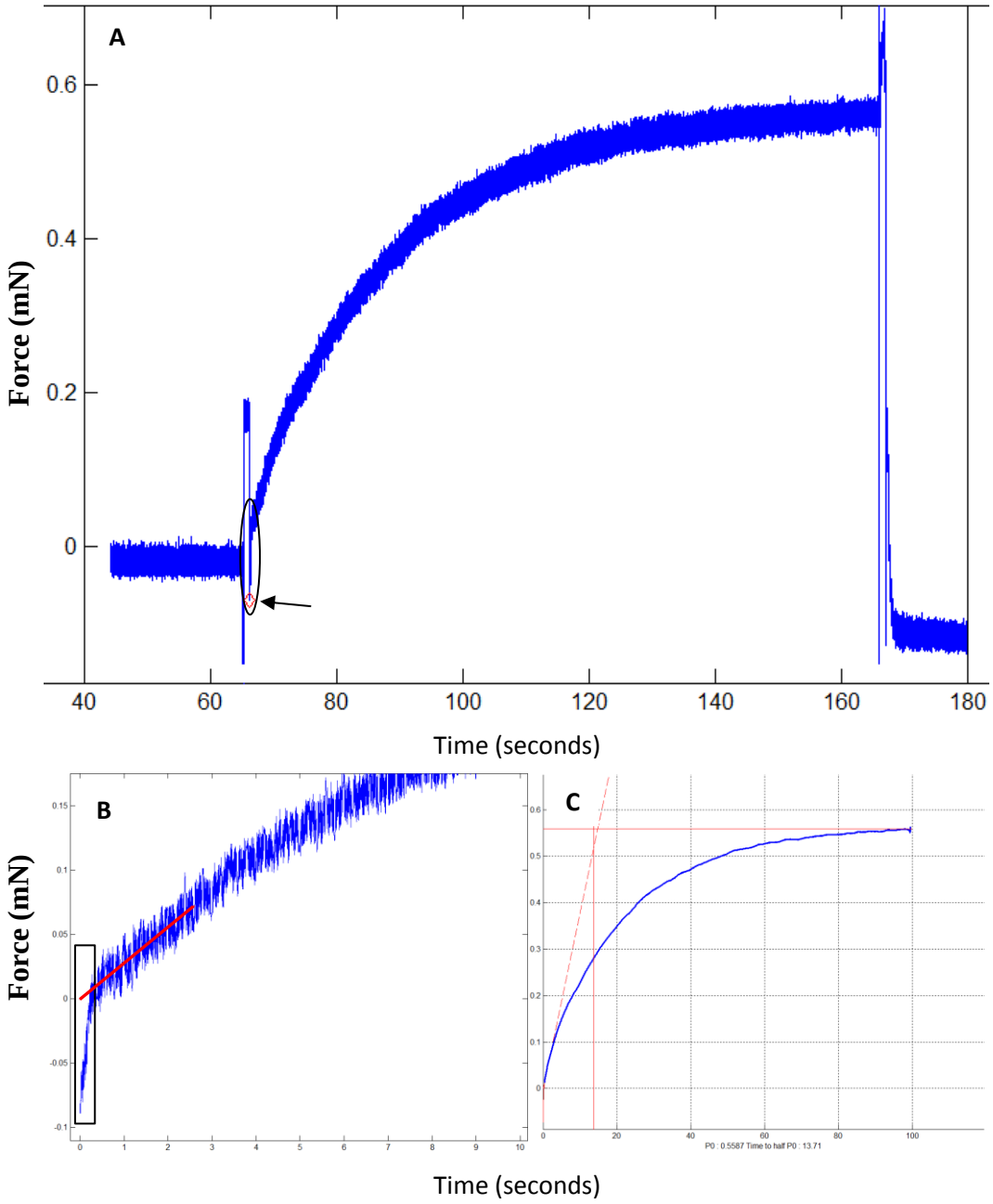


Figure 3.2. An example of the determination of the skinned fibre baseline force signal, from which P_0 could be calculated.

A: the point at which the fibre was transferred into a new experimental solution was regarded as the start point of a contraction, indicated by a red diamond.

B: A zoomed in image of the circled section in A. A red line was from the point of 10% peak force signal and extrapolated backwards along the slope of the force trace. The point at which the extrapolated line reaches the x-axis of the contraction start point (pictured in A) is regarded to be the baseline force signal. The signal artefact, lasting ~300ms, which was excluded is outlined in a black box.

C: Once the baseline has been established the signal was smoothed in Matlab and the P_0 (indicated by the horizontal red line) and the t_{50} (indicated by the vertical red line) are calculated. The dashed red line is an extrapolated version of that pictured in B.

tissue containing numerous fibres in standard Laemmli sample buffer (LSB) (Laemmli, 1970) using a gentleMACS dissociator (Miltenyi Biotec, Surrey, United Kingdom). Samples were heated at 100°C for 3-5 minutes. In order to obtain separation between MHC IIA and IIX, numerous experimental trials were undertaken. Several polyacrylamide gels of different acrylamide:bisacrylamide ratios were tested, as well as running buffers of different chemical compositions and protocols of either constant voltage or constant current were applied for different lengths of time.

The protocol which revealed the three MHC isoforms present in human skeletal muscle was carried out using a 'Biorad mini-protean 3 cell' apparatus. A 0.75mm thick, 4% stacking gel with an 8% resolving gel both containing 30% glycerol were used (Talmadge and Roy, 1993). Running buffer from the protocol of Blough et al. (1996) was used, which utilised β-mercaptoethanol in the upper running buffer. Over a 26-hour period, a constant current of 4mA was set until the bromophenyl blue marker had migrated into the separating gel, at which point the current was increased to 8mA. Gels were subsequently stained using a commercially available Coomasie blue stain (Novex SimplyBlue safe stain, LC6060) to reveal the MHC bands. Details of the gel (Table 8.1 and Table 8.2) and running buffer constituents (Table 8.3 and Table 8.4) are provided in in the Appendices.

Three clearly separated bands were found in one of the human control samples tested (Figure 3.3A), which were within the region of the known molecular weight of myosin (~220 kDa). Subsequent western blotting analysis was carried out to confirm the identity of the MHC isoforms (Figure 3.3B).

Briefly, proteins were transferred electrophoretically from the gel to the nitrocellulose membrane using a constant voltage of 10V for 45 minutes. The nitrocellulose membrane was incubated in blocking solution (PBS-Tween containing

0.1% tween with 5% w/v milk powder) for three hours at room temperature. Following blocking, the primary antibody was applied for 1.5 hours at room temperature, after which the membrane was washed in four to five changes of PBS-Tween containing 0.1% Tween for 45 minutes. The membrane was then incubated in the secondary antibody for one hour with gentle agitation, following which PBS-tween was used to wash the membrane using several washes over a 10 minute period. The stain was developed in ECL solution.

The Western blotting analysis was carried out by Ms. Katie O'Brien, from the Centre of Human and Aerospace Physiological Sciences at King's College London. The results confirmed the presence of the MHC I and IIA isoforms in the human control sample tested using the mammalian primary monoclonal antibodies BA-F8 (Immunoglobulin G, IgG) and Sc-71 (IgG) respectively (Figure 3.3B). Confirmation that the third band was type IIX myosin could not be achieved using the mammalian primary monoclonal antibody 6H1 (IgM). However, Bamman et al. (1999) used western blotting analysis on a polyacrylamide gel of exactly the same composition as that utilised in the present thesis, to show that the third band was human IIX myosin. Based on this information, the third band from the human control muscle sample was assumed to be IIX myosin.

In order to identify the myosin heavy chain composition of single skinned fibres, fibre segments were placed into standard Laemmli sample buffer (Laemmli, 1970) immediately following force measurements. Ten microliters of sample buffer were used per 1mm of skinned fibre length. The skinned fibre samples were then heated at 100°C for 3-5 minutes before being loaded onto a 0.75mm thick gel. Results were scanned using a flatbed scanner with high 4800x4800dpi resolution (CanoScan 900F Mark II, Canon).

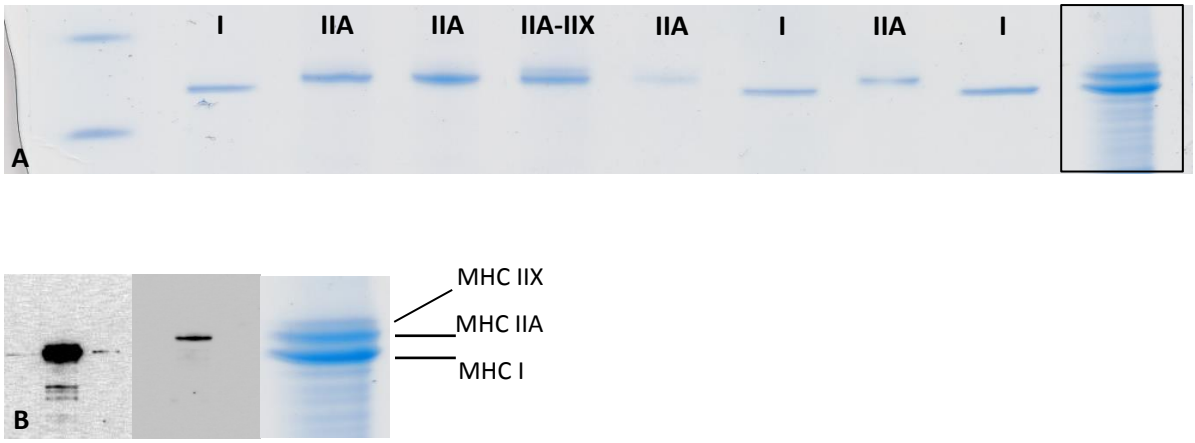


Figure 3.3. The SDS PAGE protocol used to distinguish between type I, IIA and IIX myosin heavy chain isoforms in human skeletal muscle.

A: A new protocol was developed which yielded separation of three clear bands in a sample of human skeletal muscle, in the lane outlined in a black box on the right hand side of the gel. These bands were in the range of the molecular weight of myosin, as indicated by the molecular weight marker pictured in the first lane to the left of the gel, in which the lower and upper bands correspond to molecular weights of 150 and 250 kDa respectively. The other bands pictured on the gel are from human, single muscle fibres and correspond to MHC I, MHC II or MHC IIA-IIX myosin heavy chain, as is indicated by above each band.

B: Western blotting analysis identified MHC I and IIA myosin heavy chain isoforms in the human control sample using BAF-8 and Sc-71 antibodies respectively. The MHC isoform of the third, upper band, could not be confirmed using the antibody 6H1, but was assumed to be human IIX myosin based on Wester blotting results from previous work studying human skeletal muscle which used a gel of exactly the same composition (Bamman et al., 1999).

3.12 Quantification of skinned fibre myosin content

One aim of the present thesis was to assess the mechanism of any observed age-related specific force loss by quantifying the myosin content of individual skinned fibres and correlating myosin content with skinned fibre specific force. The myosin content of an individual skinned fibre was calculated by quantifying the optical density, termed the brightness area product (BAP), of MHC bands from single fibres on SDS PAGE gels using image analysis software (ImageJ). Once the BAP of a given MHC band had been quantified, the total myosin content of a given skinned fibre was calculated assuming that the myosin content was uniformly distributed throughout the whole sample, only part of which had been loaded onto the gel. The total skinned fibre myosin content was then normalised to skinned fibre volume, which in turn was calculated from length and depth measurements (section 3.8), thus giving a measurement of skinned fibre myosin content per unit of fibre volume. The length of the skinned fibres suspended between two t-clips had been measured, so the segment of the fibre between the t-clips was carefully cut using fine dissection scissors and processed for analysis in LSB.

In order to quantify the myosin content of a given MHC band, the linear range of the myosin sample needed to be identified. This was to ensure than any differences in the BAP of individual bands were due to differences in myosin content. Three samples of a commercially available myosin standard (Sigma M7659) containing either 1µg, 3µg or 5µg of rabbit skeletal muscle myosin, were loaded onto the same gel as human, single fibre samples (Figure 3.4A). The linear range of the myosin standard was successfully identified as being in the range of 1µg-5µg of myosin (Figure 3.4B), indicating that the BAP was proportional to myosin content between 1µg-5µg. Therefore, the BAP of samples of human single fibres which have corresponding MHC bands within the 1µg-5µg range (Figure 3.4B) will be proportional to the sample's myosin content, within a

gel, and differences in myosin content between groups or any relevant trends could be observed.

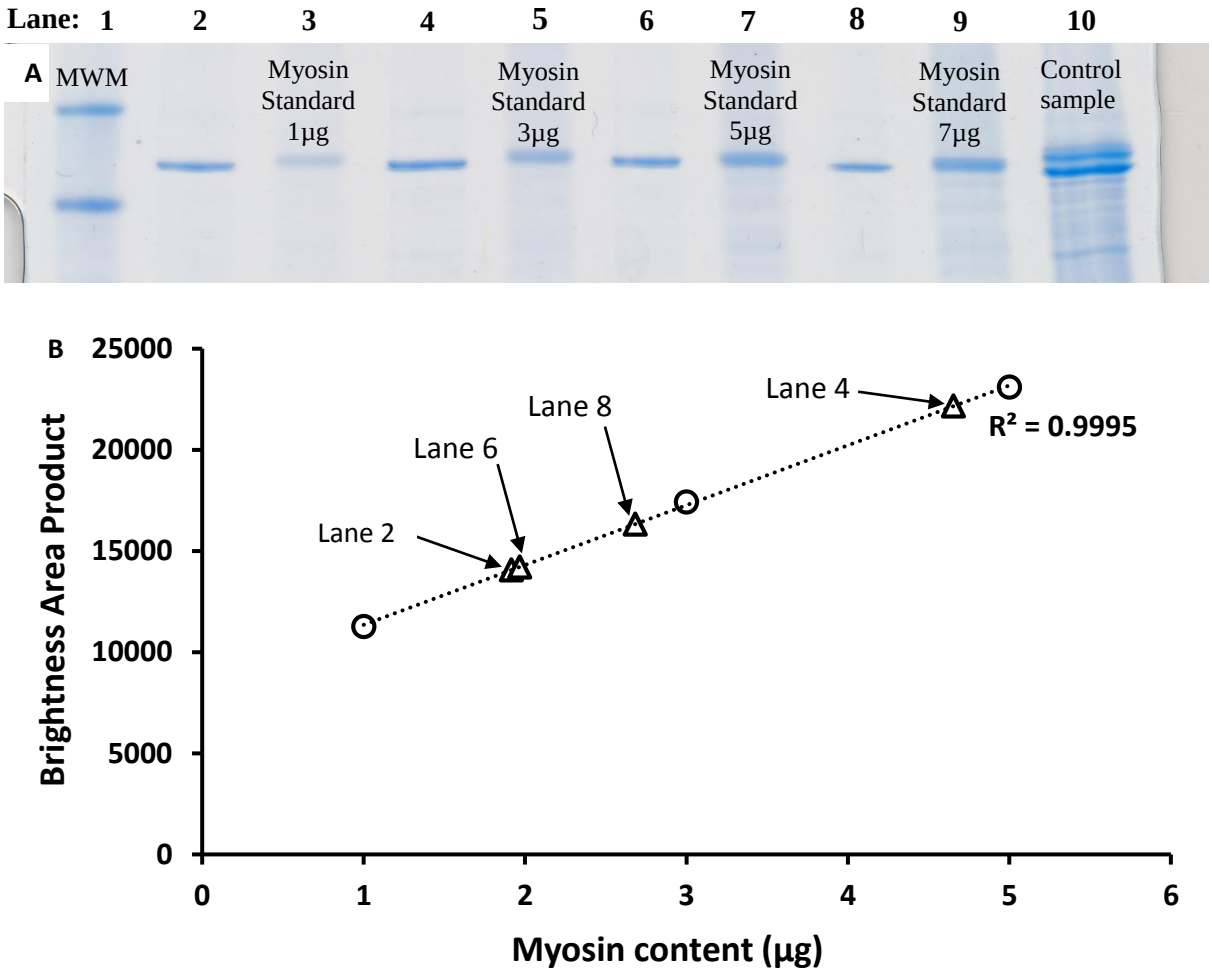


Figure 3.4. Optimisation of the myosin quantification method.

A: Myosin standard samples of 1µg, 3µg, 5µg or 7µg (clear circles) were loaded onto a gel containing human skinned single fibres, from the left, in lanes two, four, six and eight.

B: The linear range, where the optical density of a given myosin standard band increases proportionately with the sample's myosin content, was observed to be between 1µg-5µg. Therefore, the brightness area product of MHC bands from human skinned fibre samples (clear triangles) which were within the linear range of the myosin standard (lanes two, four, six and eight) could be quantified knowing that the optical density was proportional to myosin content.

4 The impact of commonly used activating solutions on skinned fibre specific force.

4.1 Extended introduction: features of activating solutions used to study skinned skeletal muscle fibres.

The different methodological practices utilised by different research groups assessed in Chapter 2 were found to account for ~30% of the variability in published specific force measurements from skinned fibres. One methodological difference which it was not possible to correct for was the effect of different activating solutions used in different publications on the reported skinned skeletal muscle fibre specific force.

Being able to carefully control the solutions surrounding skinned muscle fibres, is considered a major advantage of the skinned fibre technique and has been important in elucidating and linking biochemical processes with mechanical events (Stienen, 2000). The vast majority of research carried out to develop activating solutions which could effectively elicit a peak isometric force response from skinned fibres, occurred in the 1970s and 1980s. From the 1990s onwards the skinned fibre technique has been predominantly used to investigate the impact of a given intervention or physiological condition on muscle contraction at the level of the myofilaments. In developing activating solutions, the primary considerations made were the physiological requirements for a muscle contraction, in parallel with practical issues associated with the skinned fibre preparation, such as ensuring the uniform diffusion of a given chemical substrate across a fibre.

The primary features of a given activating solution include the precise regulation of pH, the incorporation of ATP and associated regenerating system based around CP, and a pCa which will maximally saturate a fibre with Ca^{2+} for which EGTA is used as a Ca^{2+} chelator. These chemical features must also be incorporated in such a way as to

ensure that the activating solution has an appropriate ionic strength. In this extended introduction, the rationale underpinning the aforementioned chemical features of activating solutions used to study skinned fibres will be outlined, with specific emphasis on the selection of an appropriate pH buffer to regulate the solution's pH.

4.1.1 Importance of pH regulation in skinned fibre experiments.

The study of biological reactions is complicated by the fact that a given reaction can produce thousands of times the amount of hydrogen required for maintenance of a given pH (Good and Izawa, 1972). Indeed, when the movements of cations and anions across membranes are not equal in experimental preparations such as skinned fibres, local pH changes can occur. In their highly cited work, Good et al. (1966) emphasise the importance of using efficient pH buffers by stating that "It is impossible even to guess how many exploratory experiments have failed, reactions rates have been depressed or how many processes have been distorted because of the imperfections of the buffers employed."

Controlling pH in the skinned fibre preparation can be problematic due to processes such as the hydrolysis of ATP during the attachment and/or detachment of myosin to and from actin, during skinned fibre contraction, which produces hydrogen ions. The maintenance of a neutral pH in an activating solution used, in order to regulate the internal skinned fibre pH, is important for several reasons. The regulation of Ca^{2+} in solution, necessary to elicit peak isometric force from skinned fibres, is accomplished using the metal chelator EGTA, which in turn requires stringent pH control (Murphy and Koss, 1968). Furthermore, the force-pCa relationship in both skinned crustacean (Ashley and Moisescu, 1977) and frog (Fabiato and Fabiato, 1978) skeletal muscle fibres was observed to be less sensitive at acidic compared to neutral pH ranges. The peak isometric

force measured from frog skinned fibres in an acidic (pH 6.2) activating solution was depressed compared to when measured in an activating solution of neutral (pH 7) pH (Fabiato and Fabiato, 1978). Finally, the distances between myofilaments of skinned crayfish fibres were irreversibly decreased at low pH (pH 4.4), suggesting that there may be a denaturing effect of acidic pH on myofibrillar proteins (April et al., 1972). The aforementioned findings support that selecting an appropriate pH buffer to regulate the pH of a given activating solution is important in the skinned fibre preparation.

4.1.2 Selecting an appropriate pH buffer: traits of good pH buffers

Good and Izawa (1972) specified that a desirable pH buffer has maximum solubility in water but not in other solvents, contributes as few ions as possible to a solution and has an acid dissociation constant (expressed as pKa) which is not significantly affected by a solution's temperature or ionic composition. Furthermore, having a pH buffer with a pKa close to the desired pH of a given experimental solution is beneficial because consequently a lower buffer concentration is required for a given amount of pH regulation (Good and Izawa, 1972). Finally, among other properties, good pH buffers should not react with other components in the solution.

Consistent with the criteria of a desirable pH buffer outlined above, Good et al. (1966) systematically investigated different buffers to assess their chemical properties and utility in biological research. Among the compounds which met the criteria for an efficient pH buffer, 2-[(2-Hydroxy-1,1-bis(hydroxymethyl)ethyl)amino]ethanesulfonic acid (TES) was highly recommended for use in biological research, based on its solubility in water, pKa values at different temperatures and negligible metal-buffer binding constants, among other properties. Furthermore, Good et al. (1966) explicitly state that Imidazole is not a satisfactory pH buffer, and that apart from the buffering range of

Imidazole there were few other positive characteristics reported (Good and Izawa, 1972). The identification of Imidazole as a poor pH buffer is highly relevant because the majority of studies which have measured specific force from human skinned fibres have utilised Imidazole as a pH buffer in their activating solutions. Given the clear published recommendations to *not* use Imidazole, the inclusion of Imidazole as a pH buffer in experimental solutions used to study skinned skeletal muscle fibres is surprising. The implications of the use of either TES or Imidazole as a pH buffer in activating solutions used to elicit peak isometric force from chemically skinned, human skeletal muscle fibres is discussed extensively in section 4.4.

4.1.3 The MgATP complex in activating solutions used to study skinned skeletal muscle fibres

Cross-bridge cycling during skinned skeletal muscle fibre contraction requires the hydrolysis of MgATP, which in turn requires myofibrillar ATPase activity. In the skinned fibre preparation ATP is supplied to the skinned fibre via the activating solution. Mühlrad et al. (1965) demonstrated that at different concentrations of ATP the maximal ATPase activity of myofibrils required Mg^{2+}. Furthermore, when several bivalent cations were tested, ATPase activity was found to be optimal in the presence of Mg^{2+}. The MgATP complex was also implicated in the relaxation of skeletal muscle as high ATP concentrations alone did not dissociate the actomyosin complex of myofibrils unless sufficiently high Mg^{2+} was present. The opposite was also observed, i.e. in the presence of Mg^{2+}, relaxation did not occur if the ATP concentration was sufficiently low (Mühlrad et al., 1965).

The requirement of MgATP for skinned fibre force generation was observed in skinned, single crayfish fibres, which only produced 50% of maximum force in the

absence of Mg^{2+} and ATP. Indeed, maximum skinned fibre force was only obtained in the presence of both substrates. Godt (1974) observed that the MgATP concentration utilised in a given activating solution affected the force-pCa relationship in skinned frog fibres. As MgATP was decreased, a higher pCa was required to elicit maximum tension and if too little MgATP was present rigor complexes would form, preventing tension generation from being controlled by Ca^{2+} concentration. These findings support that the MgATP complex is necessary for an optimal skinned fibre contraction.

4.1.4 The ATP regenerating system in activating solutions used to study skinned skeletal muscle fibres

Given the importance of MgATP to skinned fibre force generation, the diffusion of MgATP from the activating solution into the skinned fibre interior must be uniform. However, the hydrolysis of ATP by the myofilaments prevents the concentration of MgATP in the interior of skinned fibres from being the same as that present in the experimental solution surrounding the fibre. Godt (1974) presents calculations indicating that for a fibre 100µm in diameter, bathed in a solution containing 2mM of ATP outside the fibre, ATP will only be present in 41% of the fibre volume when the fibre is maximally activated with Ca^{2+}. This causes a gradient of differing ATP concentrations across the fibre diameter, meaning that ATP diffusion is not uniform across the fibre. Therefore, an ATP regenerating system of Creatine Phosphate (CP) and/or Creatine Phosphokinase (CPK), is typically used to regenerate ATP from ADP, to minimise issues relating to diffusion which can be caused by the intrinsic ATPase activity of skinned fibres (Ashley and Moisescu, 1977). Furthermore, the inclusion of CP and CPK was suggested to create a better representation of the normal myofibrillar environment, since both serve to maintain ATP concentration in intact muscle fibres (Godt, 1974).

The concentration of CP to be used in a given activating solution was determined experimentally in skinned frog fibres by assessing the effect of different concentrations of CP on the force-pCa relationship. Peak isometric force was not affected by different concentrations of CP, although submaximal force was reduced at CP concentrations lower than 14.5mM, indicating that below 14.5mM of CP the skinned fibre Ca^{2+} sensitivity was diminished. Therefore, 14.5mM of CP was selected as an appropriate concentration, which has also been widely employed in activating solutions used to measure specific force from human skinned fibres. Indeed, approximately 60% of the human skinned fibre specific force data sets reviewed in Chapter 2 were measured using activating solutions containing 14.5mM of CP.

4.1.5 The relationship between Calcium concentration and force in skinned fibres

The importance of Ca^{2+} in the development of force during a skinned and intact fibre muscle contraction was initially observed in the 1960s. Bianchi and Shanes (1959) tracked the Ca^{45} radioisotope during contraction of intact frog Sartorius muscle and observed that there was a direct relationship and strong agreement between the strength of the contraction twitch and the amount of Ca^{2+} entering the muscle. This evidenced a simple, direct relationship between Ca^{2+} influx and twitch height. In glycerinated rabbit psoas fibres Seidel and Gergely (1963) observed a lack of tension when fibres were in a solution containing purified ATP which was free from contaminating Ca^{2+}, which was in contrast to un-purified ATP. This pointed to a role of Ca^{2+} in the contraction of skinned skeletal muscle fibres. Indeed, adding $CaCl_2$ to solution caused an increase in tension by increasing the Ca^{2+} concentration in the experimental activating solution.

The role of Ca^{2+} in skinned fibre force production was also implicated indirectly through observations made using EGTA, a chelating agent used in skinned fibre solutions

due to its selective affinity for Ca^{2+} ions compared to other metal ions such as Mg^{2+}. Ebashi and Ebashi (1962) showed that the relaxing ability of different metal chelators was linearly proportional to their Ca^{2+} binding capacity, implicating a role of Ca^{2+} in force development. Indeed adding EGTA was found to inhibit Ca^{2+} elicited contractions in glycerinated psoas fibres (Filo et al., 1965).

Since Ca^{2+} had been identified as having a critical role in eliciting skinned fibre force production, several publications in the mid-1970s to early 1980s assessed the impact of different Ca^{2+} concentrations in activating solutions, on force production. The force-pCa relationship was studied in parallel with varying concentrations of another substrate in solution, such as CP (Godt, 1974), Mg^{2+} (Donaldson and Kerrick, 1975, Stephenson and Podolsky, 1977), or K^{+} and ionic strength (Ashley and Moisescu, 1977). The impact of chemical skinning on the force-pCa relationship (Orentlicher et al., 1974), and Ca^{2+} kinetics in the process of tension development were also studied (Moisescu, 1976). These studies all observed that peak skinned fibre force was generated in the presence of high concentrations (pCa 4.5-5) of Ca^{2+}. Fuchs and Fox (1982) determined the relationship between skinned rabbit psoas fibre Ca^{2+} saturation and force, reporting that peak force was elicited when skinned fibres were fully saturated with Ca^{2+}, which in turn occurred in solutions at pCa 5. Therefore, investigations studying the peak isometric force response of skinned skeletal muscle fibres use activating solutions at pCa 4.5 in order to provide an excess of Ca^{2+}, to ensure peak force is achieved, which corresponds to a total Ca^{2+} concentration of 30mM (Ferenczi et al., 1984). Indeed, all the studies assessed in the systematic review presented in Chapter 2 measured specific force from human skinned skeletal muscle fibres using activating solutions at pCa 4.5.

Maintaining a constant Ca^{2+} concentration throughout a given skinned fibre can be complicated by the generation of H^{+} from the hydrolysis of ATP during contraction.

The movement of protons across a skinned fibre can cause local pH changes which affect the apparent binding constant of Ca^{2+} to the Ca^{2+} buffer (EGTA) utilised. Therefore, Moisescu and Thieleczek (1978) developed a method to rapidly equilibrate the Ca^{2+} concentration between the activating solution used and the skinned fibre interior. This involved incubating a fibre in a pre-activating solution with a low EGTA concentration, followed by rapid immersion into an activating solution containing a high Ca^{2+} buffering capacity, causing the desired ratio between CaEGTA and EGTA to be attained more quickly. Indeed, based on the light intensity of aequorin, the Ca^{2+} sensitive photoprotein, the time course for the Ca^{2+} concentration of the whole skinned fibre to reach 90-95% of that in the activating solution was 100-150ms. The technique of utilising a pre-activating solution is now common practice.

4.1.6 The impact of the experimental solution ionic strength on skinned fibre force.

The ionic strength of a given activating solution used to study skinned skeletal muscle fibre contraction is important, since at sub-physiological temperatures high ionic strengths have been shown to reduce P_0. In experiments conducted at room temperature (20-22°C), a reduction in Ca^{2+} induced force from skinned frog fibres transferred from solution of 170mM to 330mM ionic strength was observed. Furthermore, force was irreversibly lowered when fibres were immersed in solutions of 400-500mM ionic strength. The force response of skinned frog fibres was in high agreement with that observed in intact frog fibres, indicating that the decline in force was likely due to an elevated internal ionic strength, causing the force generation of myofibrillar proteins to be compromised (Gordon et al., 1973). In mechanically skinned soleus and extensor digitorum longus (EDL) rat fibres and Iliofibularis toad fibres, Ca^{2+} sensitivity was reduced as the ionic strength of the activating solution increased. Furthermore, P_0 decreased with increasing ionic strength in all fibres studied (Fink et al., 1986). The mechanism of these observations is discussed further in section 4.4.5.5. Due to the negative effect of high ionic strength on skinned fibre force, a physiological value of approximately 200mM is typically employed (West et al., 2005).

4.1.7 Aims

Substantial literature exists which has investigated how different chemical substrates in solution affect the skinned fibre contractile response. Even so, a consensus does not exist as to the chemical composition of an activating solution which elicits the optimal skinned fibre contractile response with regard to force generation. This has resulted in different research groups using different activating solutions to study the contractile properties of skinned fibres. Therefore, in order to assess whether the different activating solutions used by different research groups contributes to the variability in reported specific force results, the aims of the present study were to:

1. Quantify the effect that two different and commonly used activating solutions have on the contractile response of chemically skinned, human skeletal muscle fibres. Each activating solution tested is used by a different research group that routinely takes mechanical measurements from skinned skeletal muscle fibres (Larsson and Moss, 1993, West et al., 2013).
2. To systematically identify the cause of any observed differences in skinned fibre contractile response elicited by the two different activating solutions, by isolating the effects of individual chemical components.

4.2 Methods

4.2.1 Subjects

One young, healthy, male participant (24 years old) provided informed consent to volunteer for the study, which was approved by the King's College London ethics committee and ran in accordance with the declaration of Helsinki (1964).

4.2.2 Biopsy and preparation of muscle samples for mechanical experiments

A muscle biopsy was taken from the Vastus Lateralis using a Bergstrom biopsy needle with suction (Bergstrom, 1962) (section 3.3), and bundles of approximately fifty to one hundred fibres were chemically skinned (section 3.4). All bundles were processed for long-term storage (section 3.5) and subsequently de-sucrosed before being prepared for experimentation (section 3.6), to ensure that all skeletal muscle fibres had undergone the same treatment prior to force measurements being taken. Individual fibres were dissected from a bundle, t-clipped and mounted to the experimental set up (section 3.8), sarcomere length was set to an optimal length of 2.75μm and the dimensions of a given skinned fibre were measured. Skinned fibre CSA was calculated assuming a cylindrical cross-section, without correcting for the swelling which occurs upon skinning (section 3.8).

4.2.3 Force measurements from chemically skinned skeletal muscle fibres

Skinned fibres were transferred from a relaxing (pCa 9.0) to a pre-activating solution, followed by an activating solution (pCa 4.5) (section 3.9) where the fibres contracted until a plateau in force signal was reached, before being transferred back into relaxing solution. Peak isometric force (P_0) was classified as the calculated baseline force signal of the fibre in the activating solution subtracted from the maximum force signal recorded (section 3.10).

4.2.4 Identifying the fibre type of individual fibres using SDS-PAGE

SDS PAGE was used to identify the MHC isoform composition of individual skinned fibres based on their myosin heavy chain isoform expression, as described in section 3.11. A sub-set of the skinned fibres tested in the present study were stored at -20°C for up to fourteen months. Unfortunately, when fibre typed these samples yielded no bands, which was attributed to protein degradation over time. The data from these fibres has been grouped together and they are referred to as fibres of 'unclassified fibre type.' Subsequently all fibres tested following this were stored at -80°C and were analysed within two months. The fibres yielded clear bands, enabling the differentiation of different fibre types (section 3.11).

4.2.5 Comparing the effect of solutions A and B on specific force elicited from the same fibre.

As the same skinned Vastus Lateralis fibres were being compared in two different activating solutions, a protocol was developed in order to ensure that any observed results were due to the contractile properties of the skinned fibres tested, and not the order in which the activating solutions were used. Furthermore, repeated contractions are known to potentially cause damage to skinned fibres. Therefore, a means of verifying that the structural integrity of the skinned fibres tested remained intact was necessary. A protocol in which five maximal isometric contractions were elicited from each fibre tested was used to differentiate between the effects of the two different activating solutions used, referred to as 'solution A' and 'solution B.' The chemical constituents of solutions A and B are detailed in Table 3.2, while the constituents of all other solutions used in this study are in Table 4.1.

P_0 was measured from each fibre five times: two successive contractions were measured in each activating solution, followed by a final contraction in the initial solution. The order in which the activating solutions were used was allocated randomly. Measuring fibre force in each activating solution twice ensured consistency in the data, while the final contraction served as a control value. If the force of the final contraction was not sufficiently consistent with that of the second contraction, the inference made was that the force produced was due to the fibre condition and not the effect of a given activating solution.

Skinned fibres were tested if they did not exhibit any damage upon visual inspection, such as tears or nicks along the fibre length or diameter. Data were included for analysis if there was a coefficient of variation of < 28% in specific force within a pair of contractions measured in the same activating solution. 28% was selected because it was the value which allowed the inclusion of as many data points as possible without affecting the correlation (R^2) between the mean specific force of a pair of contractions from a skinned fibre in solution A vs solution B. It was observed that the force produced by a fibre during its second contraction was typically greater than the first. Therefore the force produced during the first contraction was discounted from data analysis.

4.2.6 Examining the cause of the different contractile responses elicited by the two solutions.

Solutions A and B had three obvious differences in chemical composition which warranted further testing to isolate the effects of each chemical. The differences were 1) the use of Imidazole in solution A or TES in solution B as a pH buffer, 2) the use of Glutathione (GL) in solution B which was not used in solution A and 3) the use of Potassium Chloride (KCl) or Potassium Propionate (K-prop) to adjust the ionic strength of solutions A and B respectively (Table 3.2). The chemical compositions of solutions A

and B were manipulated in order to isolate the effect of specific compounds which were different between the two solutions. Hence, the effect any of the aforementioned compounds had on the contractile response of skinned fibres could be identified.

A purpose written computer programme designed in Matlab by Professor Roger Woledge (section 3.10) was used to manipulate the chemical composition of solution B in order to make modified versions of both solutions A and B. Two modified versions of solution B were made, and one modified version of solution A was made, keeping the concentrations of all chemical constituents essentially the same:

i) In the first modified activating solution an optimal concentration of Imidazole (20mM) was substituted for TES (solution B1). This was to assess the effect of using TES or Imidazole in a given activating solution on the skinned fibre contractile response. The investigator was blinded for the comparison of specific force elicited by activating solution B compared to B1.

ii) In the second modified activating solution tested, GL was excluded entirely from solution B, to assess the effect of GL on the contractile response of skinned skeletal muscle fibres. This solution is referred to as solution B2.

iii) The third modified activating solution made was a variation of solution A, named solution A1, which was made using K-prop instead of KCl to adjust the ionic strength. Therefore, the impact of K-prop (in solution B) or KCL (in solution A) on the contraction of skinned skeletal muscle fibres could be assessed.

The two activating solutions, A and B, and the modified activating solutions, B1, B2 and A1, were tested using the previously described 5 contraction protocol. The

optimum concentration of Imidazole for skinned fibre specific force production, which was used in solution B1, had been previously determined experimentally using a dose-response curve. These findings are illustrated in Figure 4.6 and discussed in section 4.4.5. The optimum concentration of TES for skinned fibre specific force production was also determined using a dose-response curve, described below.

The effect of TES and Imidazole on the contractile response of skinned fibres was examined more closely by plotting a dose-response curve for each compound. New versions of solution B were made each containing either 20, 40, 60, 80 or 100mM of TES. A pair of contractions was elicited from a skinned fibre in each of the five activating solutions which were used in a random order. The data was accepted if a high consistency in force production was observed in each solution. A comparison using the previously described five contraction protocol was also made between solution B and another version of solution B from which all TES was removed, which was plotted on the dose-response curve Figure 4.6.

To plot the Imidazole dose response curve solution B was made containing 0, 5, 10, 15, 20, 40, 60, 80 or 100mM of Imidazole. Two dose response curves were plotted between Imidazole concentrations of 0mM to 20mM and 20mM to 100mM. Both dose-response curves are plotted on the same figure as the TES dose response curve for comparative purposes. The concentrations of the other chemical constituents in the new versions of solution B remained the same apart from minor differences in K-prop so as to adjust the ionic strength to 200mM. In two solutions, containing 80 or 100mM of Imidazole, the ionic strength had to be adjusted to 210mM and 220mM respectively to accommodate the increased Imidazole concentration. Details of the constituents of all solutions are given in Table 1. A twelve contraction protocol was employed for the dose-response curve of Imidazole. A pair of contractions was elicited from a skinned fibre in

each of 5 activating solutions used in a random order, followed by a final pair of contractions in the initial activating solution which served as an internal control.

4.2.7 Proton nuclear magnetic resonance spectroscopy (^{1}H NMR)

In order to identify whether the inclusion of Imidazole or TES in solutions B and B1 respectively, caused the formation of any compounds affecting the skinned fibre contractile response, a spectrum of the compounds containing H^+ nuclei was recorded using proton nuclear magnetic resonance spectroscopy (^{1}HNMR). Samples of 475µl of each solution were mixed with 25µl of deuterium oxide (D_2O) in ependorph tubes before being transferred to 5mm diameter NMR tubes (Willmad-LabGlass). A 'blank' sample containing only the distilled water (H_2O) used to make the experimental solutions in the present thesis, and no other chemicals apart from 25µl of D_2O, was also tested to assess the purity of the distilled H_2O.

1D ^{1}H NMR spectra were recorded at 298K and 700 MHz (representing the ^{1}H frequency at that magnetic strength) on a Bruker Avance III 700 NMR spectrometer. The signal from the distilled water used to make up solutions B and B1 was suppressed using a 1D NOESY pulse sequence (noesygppr1d). Samples were scanned thirty-two times with a relaxation delay of four seconds between each scan. Spectral width was set to 20.5ppm.

Data was processed and analysed in the manufacturer's software (Topspin v.3.2). The raw spectra obtained were treated with an exponential multiplication window function (line broadening of 0.3 Hz), prior to being processed using a Fourier transformation. Spectra were calibrated to an external reference (4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) at 0.00 ppm).

4.2.8 Statistics

The mean specific force elicited from each contraction of the five contraction protocol was compared *within* the specific force data collected in solution A or solution B using a one-way ANOVA. The mean specific force data collected from a given fibre in the activating solutions used for the dose-response curves for TES or Imidazole were also analysed using a one-way ANOVA.

Mean specific force measurements from pairs of contractions of the same fibres in two given, different activating solutions tested using the previously described five contraction protocol, were analysed using a paired t-test. The corresponding mean t_{50} values were also analysed using a paired t-test. The experiments for which the data were analysed using a paired t-test were: solution A vs B; B vs B1; B vs B2; A vs A1 and solution B vs solution B containing no TES. Mean specific force measurements from pairs of contractions of two different sets of fibres tested in either solution B containing no TES or solution B containing no Imidazole, were analysed using an independent t-test.

Correlations between t_{50} and skinned fibre CSA and between specific forces elicited by solution B or B1 from different fibre types were assessed using type I linear regression.

Solution	ATP (mM)	PCr (mM)	EGTA (mM)	CaEGTA (mM)	$MgCl_2$ (mM)	$CaCl_2$ (mM)	TES (mM)	Imidazole (mM)	K-propionate (mM)	KCl (mM)	Glutathione (mM)	Ionic Strength (mM)
Relaxing (pCa 9.0)	4.65	14.50	7.00		5.46	17.70 (µM)		20.00		77.63		200
Pre-activating	5.20	19.60	0.50		5.42	16.00 (µM)		20.00		79.25		200
Activating (A1)	4.76	14.50		14.00	5.27	15.00 (µM)		20.00	81.70			200
Activating (B1)	4.65	20.00	0.17	24.83	7.7		60.00		26.10			200
Activating (B2)	4.65	20.00	0.17	24.83	7.7			60.00	1.70		10.00	200
TES dose response	4.65	20.00	0.17	24.83	7.7		20.00		23.70		10.00	200
TES dose response	For TES concentrations of 40, 60, 80 and 100mM there were corresponding K-prop concentrations of 19.90, 16.00, 12.20 and 8.40mM. The concentrations of all other constituents remained the same.											
Imidazole dose response	0.47	20.00	0.17	24.83	7.70			20.00	18.90			200
Imidazole dose response	For Imidazole concentrations of 0, 5, 10, 15, 40, 60, 80 and 100mM there were corresponding K-prop concentrations of 27.60, 25.40, 23.20, 21.10, 10.30, 1.70, 3.00 and 4.30mM. At Imidazole concentrations of 80 and 100mM the ionic strength had to be increased to 210mM and 220mM respectively. The final difference between solutions of different Imidazole concentrations was that solutions containing 0, 80 and 100mM had corresponding ATP concentrations of 4.64, 4.68 and 4.70mM.											

Table 4.1. The concentrations of the chemical constituents in the relaxing and pre-activating solutions used, as well as all modified versions of solution B and the one modified version of solution A.

4.3 Results

4.3.1 Specific force elicited by solutions A and B

Specific force was similar between contractions in solution A or solution B ($p > 0.05$) but was found to be consistently higher in solution B for all fibre types examined (Figure 4.2). Therefore, the specific force produced by a skinned fibre was dependent on the specific activating solution used and was not affected by the order in which solutions A and B were utilised.

The mean specific force measured from a pair of contractions of the same fibre was significantly ($p < 0.05$) greater for MHC I fibres ($n = 10$) activated in solution B (162 ± 68 kPa) compared with solution A (97 ± 43 kPa). The same significant trend ($p < 0.05$) was observed for fibres of unclassified MHC isoform ($n = 33$) in solution B (105 ± 43 kPa) compared with solution A (66 ± 32 kPa). However, while there was a trend for MHC IIA ($n = 10$) fibres to produce a higher specific force in solution B (135 ± 34 kPa) compared with solution A (122 ± 41 kPa) the effect was not statistically significant ($p > 0.05$) (Figure 4.3).

4.3.2 Time to half peak tension (t_{50}) in fibres contracting in solutions A and B

A major difference in the contractile response of skinned fibres activated in solutions A & B was the t_{50} (Figure 4.4). Skinned fibre t_{50} was significantly ($p < 0.05$) longer in solution A compared with the same fibres activated in solution B, requiring 9.5 ± 5.2 seconds and 1.7 ± 0.8 seconds respectively in fibres of unclassified MHC isoform. t_{50} was significantly shorter ($p < 0.05$) in MHC I fibres contracting in solution B (2.27 ± 1.4 seconds) compared with solution A (7.7 ± 5.6 seconds), as well as in MHC IIA fibres contracting in solution B (1.12 ± 0.6 seconds) compared with solution A (4.34 ± 3.7 seconds). Interestingly, within solution B the t_{50} of MHC IIA fibres was significantly

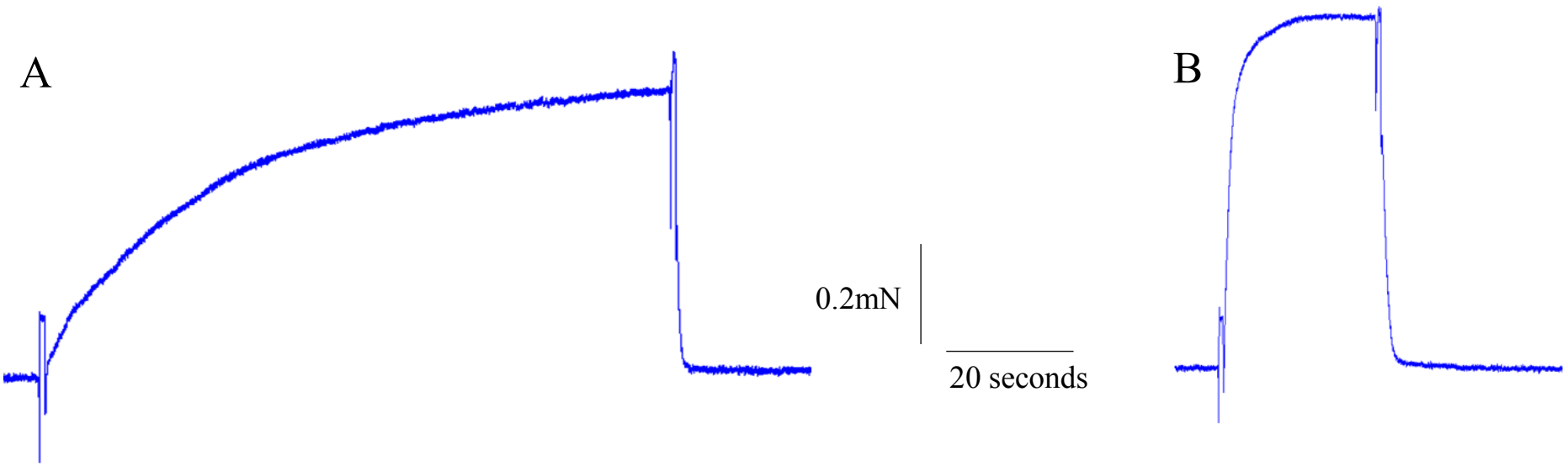


Figure 4.1. A visual representation of a force trace obtained from the same skinned fibre contracting in solution A (A) and solution B (B). The vertical axis represents force measured in mN and the horizontal axis represents time measured in seconds. The time required to reach P_0 was longer in solution A, and P_0 was higher in solution B.

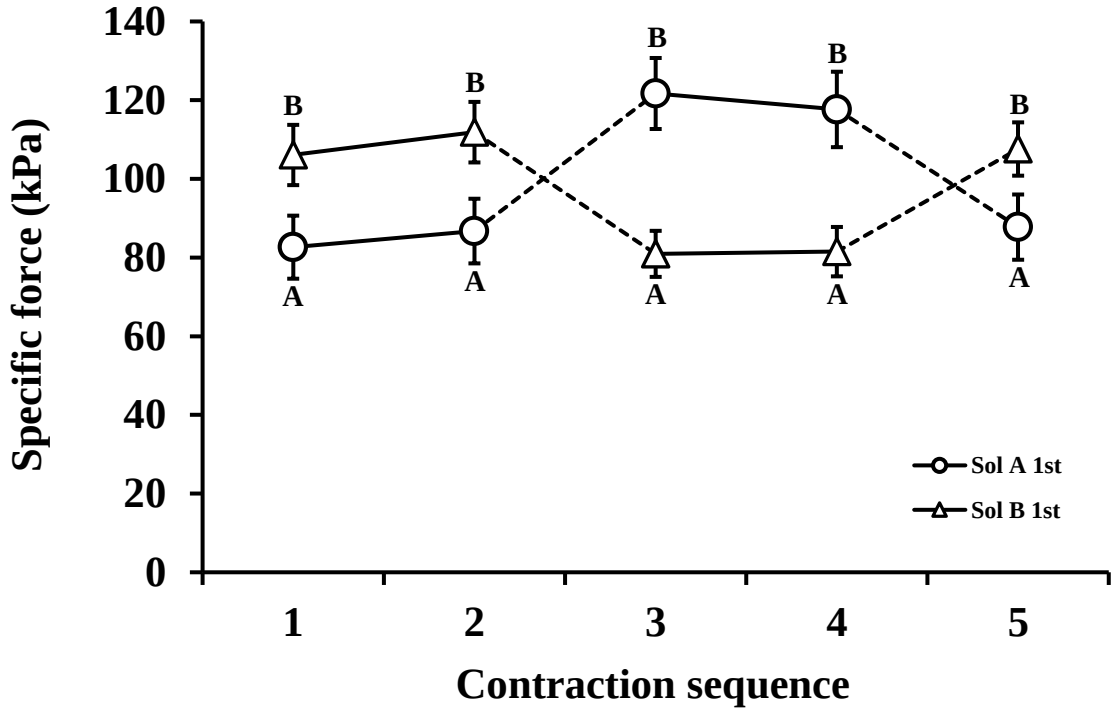


Figure 4.2. Activation protocol used in the present study.
The solution used to elicit the first contraction was selected randomly. The final contraction was measured in the same solution as the first contraction and served as an internal control measurement. Fibres (n = 55) were caused to contract in both solution A or B for two successive force measurements to ensure consistency of skinned fibre contractile performance in each solution. There was no significant ($p > 0.05$) difference between any of the specific forces measured in the same activating solution, irrespective of the order of contraction, for all fibre types examined. Values are plotted as means ± SEM.

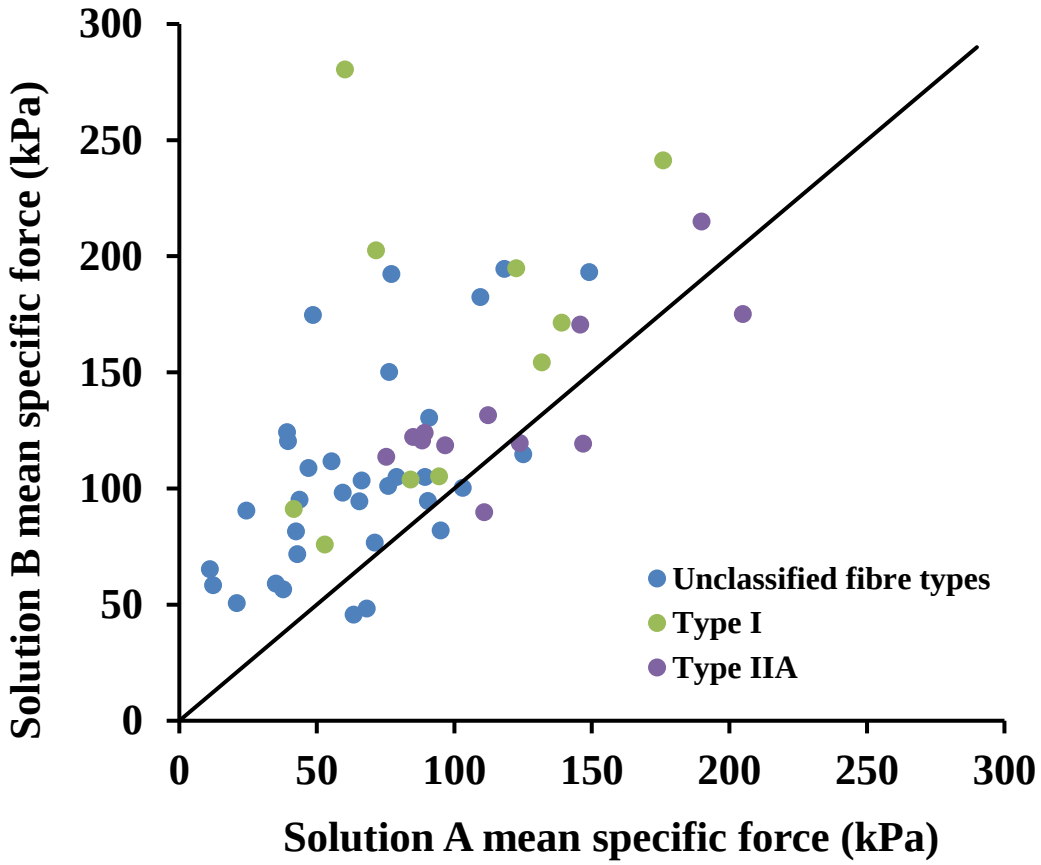


Figure 4.3. Specific force measured from the same individual fibres activated in solution B (y-axis) vs solution A (x-axis).
Each data point represents the mean value of a pair of specific force measurements taken in each activating solution. The solid black line represents the line of identity. Paired t-tests indicated that specific force was significantly higher ($p < 0.05$) in solution B compared with solution A, for MHC I fibres and fibres of unclassified fibre type, but not for MHC IIA fibres ($p > 0.05$).

shorter than the t_{50} of MHC I fibres, whereas in solution A the same trend was statistically insignificant ($p > 0.05$).

In order to assess whether diffusion of solution A or B into the skinned fibre interior may have caused the observed trends in t_{50}, the t_{50} was correlated against the fibre CSA. An R^2 of 0.36 and 0.31 was observed for MHC IIA fibres in solutions A and B respectively. However, these R^2 values were skewed by a single outlying data point, which when removed reduced the R^2 values to 0.0046 and 0.025 in solutions A and B respectively. Therefore, it was concluded that there was no correlation observed between the t_{50} in solutions A (Figure 4.5i) or B (Figure 4.5ii) and skinned fibre CSA for all fibre types, as evidenced by the weak R^2 values.

4.3.3 Chemical differences between solutions A and B and their effect on skinned fibre contractile properties

1) TES vs Imidazole dose response curves

A dose-response curve for TES was constructed using five activating solutions, all of which were modified versions of solution B (already containing 60mM of TES), with TES concentrations of 20, 40, 80 and 100mM, and their effect on skinned fibre specific force was compared. An optimum specific force was obtained using a solution containing 60mM of TES (Figure 4.6), but there was no significant difference ($p > 0.05$) in specific force observed at the other TES concentrations tested between 20-100mM. Specific force was significantly lower ($p < 0.05$) in a solution containing no TES compared with solution B, which contains 60mM of TES. The specific force data from the TES dose response curve are expressed relative to the peak specific force measured in an activating solution containing 60mM of TES, i.e. as a proportion of the maximum specific force value.

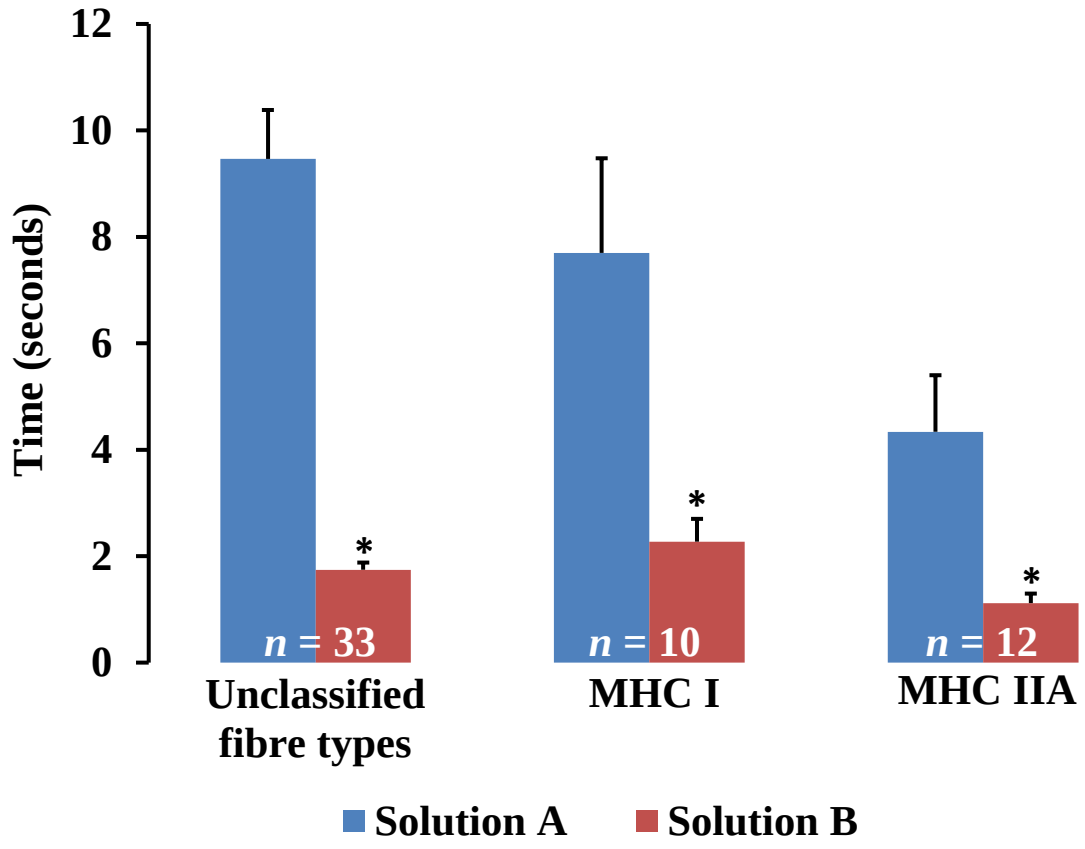


Figure 4.4. The mean time to half peak tension (t_{50}) measured from the same individual fibres activated in solution A vs solution B.
Data are displayed as means ± SEM. t_{50} was significantly ($p < 0.05$) longer in solution A compared with solution B for all fibre types examined.

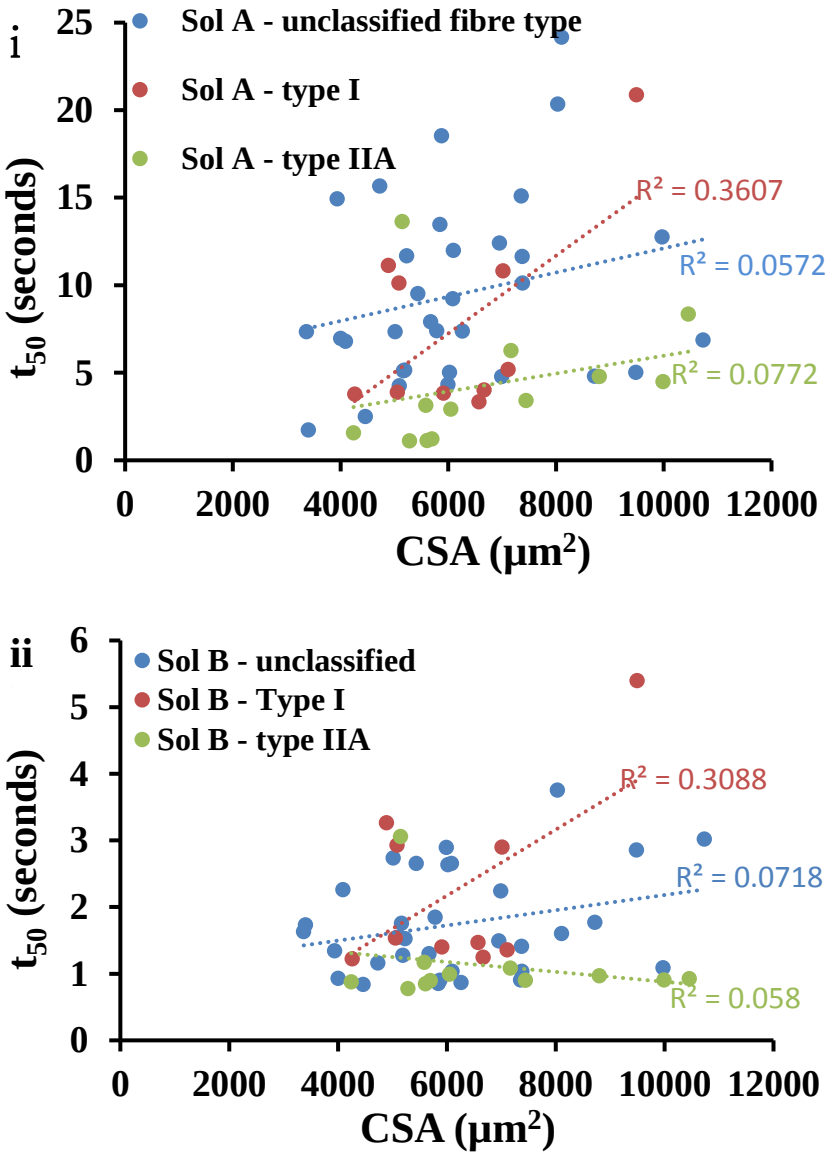


Figure 4.5. t_{50} vs CSA of skinned fibres.
i): in solution A. ii): in solution B. Blue circles represent fibres of unclassified fibre type, red circles represent MHC I fibres and green circles represent MHC IIA fibres.

Nine modified versions of solution B, containing either 0, 5, 10, 15, 20, 40, 60, 80 or 100mM of Imidazole were used to obtain a dose-response curve of the sensitivity of skinned fibre specific force to Imidazole. No significant differences in specific force elicited from skinned fibres using activating solutions containing 0, 10, 15 or 20mM of Imidazole were observed, although peak specific force was recorded in the activating solution containing 10mM of Imidazole. Specific force was significantly lower in an activating solution containing 5mM of Imidazole compared to 0, 10, 15 and 20mM of Imidazole. Specific force elicited in activating solutions containing greater than 20mM of Imidazole was reduced, exhibiting a negative linear relationship between increasing Imidazole concentration and specific force (Figure 4.6). Specific force was significantly lower ($p < 0.01$) in activating solutions containing 80mM and 100mM of Imidazole compared with 20mM. The specific force data from the Imidazole dose-response curve are expressed relative to the specific force measured in the activating solution containing 20mM of Imidazole, which is the concentration most frequently used in the skinned fibre literature.

4.3.4 The impact of TES and Imidazole on specific force at their optimal concentrations.

Having identified the concentrations of TES (60mM) and Imidazole (20mM) which elicit the greatest specific force from skinned fibres, it was of interest to compare the effect that each compound had on specific force at their optimal concentrations in solution. Given that there was no significant difference between specific force elicited in solutions containing 10mM and 20mM of Imidazole, and the fact that the latter concentration is used by the majority of the skinned fibre literature in human skeletal

muscle, Solution B (containing 60mM of TES) was compared against solution B1, containing 20mM of Imidazole.

The mean specific force measured in activating solution containing 20mM of Imidazole was significantly lower ($p < 0.05$) than in solution B containing 60mM of TES, for fibres of unclassified MHC isoform, MHC IIA and MHC IIA-X fibres. SDS-PAGE revealed that only two fibres tested in this experiment were MHC I fibres and although specific force was lower in solution B1 containing 20mM of Imidazole, this effect was not statistically significant ($p > 0.05$) (Figure 4.7A). The t_{50} of fibres contracting in solution B compared to solution B1 were not significantly different for all fibre types studied (Figure 4.7B).

Furthermore, a scatter diagram of the specific force data from each fibre activated in solutions B and B1 yielded high correlations for fibres of unclassified fibre type ($R^2 = 0.93$), MHC IIA fibres ($R^2 = 0.99$) and MHC IIA-X fibres ($R^2 = 0.84$) (Figure 4.8). This indicates that the depressive effect on specific force of Imidazole, in activating solution B1, was consistent across different fibre types. However, a correlation was not calculated for MHC I fibres since there were too few samples.

^{1}H NMR spectra of solutions B and B1 corresponded to the compounds expected based on their known chemical compositions. There was no indication of reaction products as a result of the use of either TES or Imidazole. The distilled water sample did not exhibit any compounds other than H_2O.

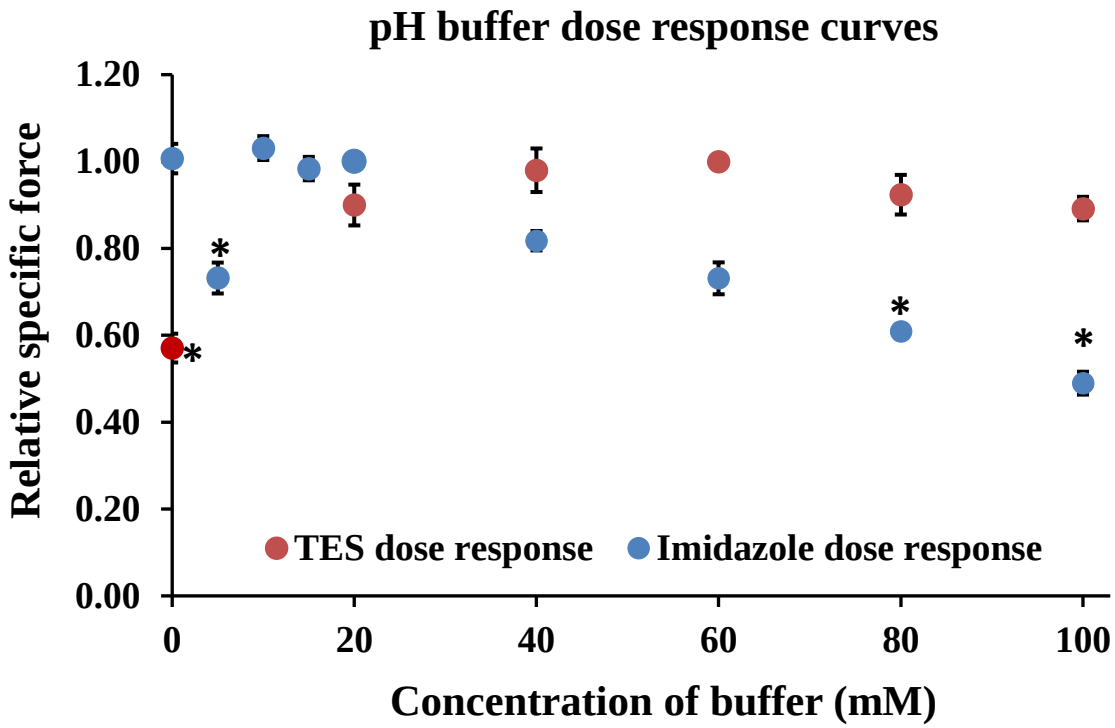


Figure 4.6. Specific force dose response curves for both TES (red circles) and Imidazole (blue circles) pH buffers.
Skinned fibres were activated in solutions containing different pH buffer concentrations in a random order, but data are presented in order of increasing buffer concentration on the x-axis. Data are mean values normalised to the peak specific force observed ± SEM. No significant difference ($p > 0.05$) in specific force was observed when fibres contracted in solutions containing different concentrations of TES ($n = 9$). In a separate experiment specific force was significantly ($p < 0.05$) lower in a solution containing 0mM TES compared with a solution containing an optimal amount of TES (60mM) ($n = 8$), displayed on the current figure. The Imidazole dose response curve was carried out in two separate experiments, the first between 20-100mM ($n = 9$) and the second between 0-20mM of Imidazole ($n = 12$). Specific force was plotted relative to force at 20mM of Imidazole, the concentration most widely used in the literature. 5mM, 80mM and 100mM of Imidazole had a significantly ($p < 0.05$) negative impact on specific force.

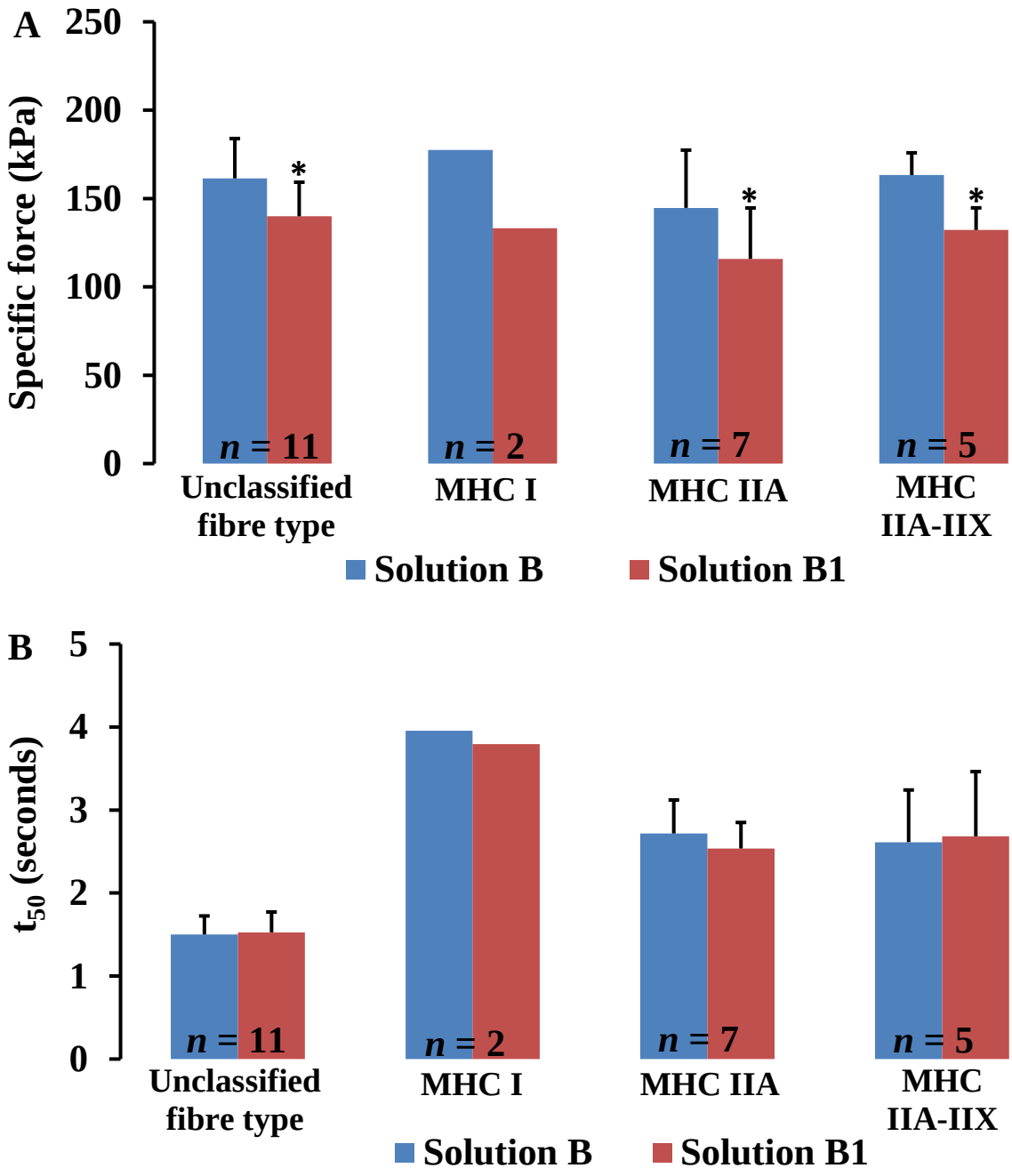


Figure 4.7. Specific force and t_{50} of skinned fibres activated in solutions B and B1. **A:** The mean specific force of the same individual fibres activated in solution B vs solution B1. Solution B contained an optimal (60mM) concentration of TES, while solution B2 contained an optimal concentration (20mM) of Imidazole as a pH buffer. Specific force was significantly ($p < 0.05$) lower in the same fibres contracting in solution B1 compared with solution B in all fibre types examined except for MHC I fibres ($p > 0.05$), as indicated by the asterisks. **B:** The mean t_{50} from the same individual fibres activated in solution B vs B1. No significant differences were observed. All data are displayed as means ± SEM.

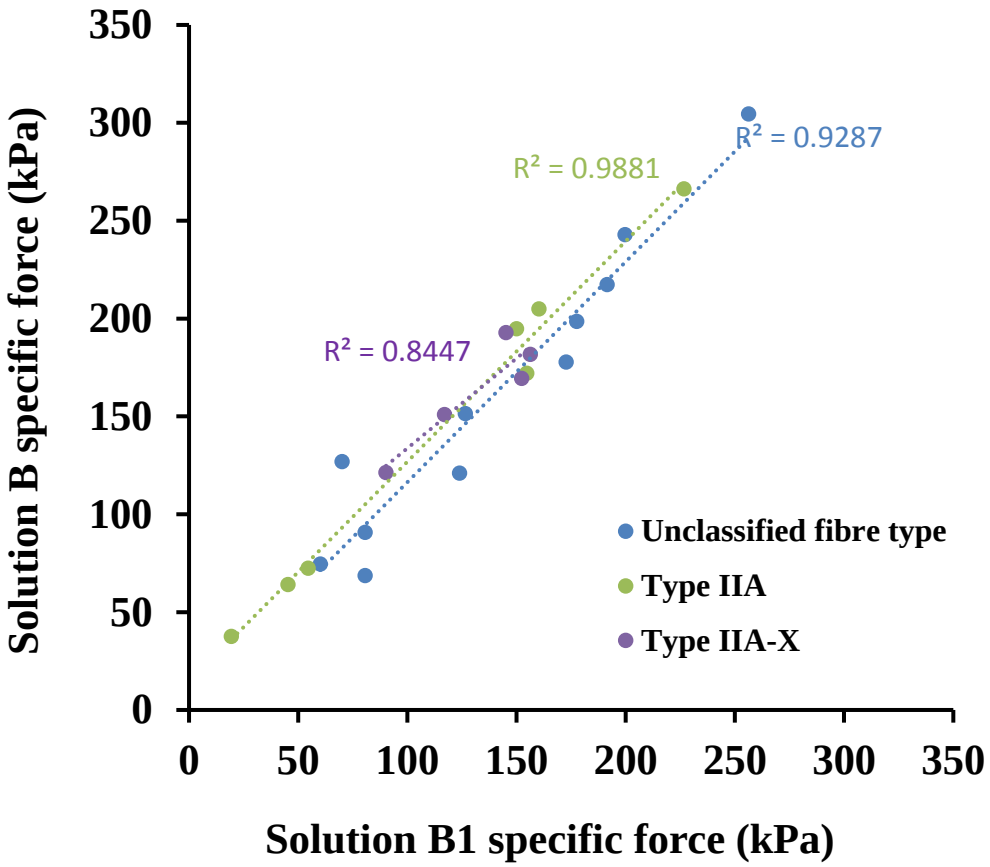


Figure 4.8. The correlation between specific force produced by the same fibres in solution B (containing 60mM TES) and solution B1 (containing 20mM Imidazole). Blue circles represent fibres of unclassified type, green circles represent type IIA fibres and purple circles represent MHC IIA-X fibres. High R^2 values of 0.84, 0.92 and 0.99 were observed for MHC IIA-X, unclassified fibre types and MHC IIA fibres respectively.

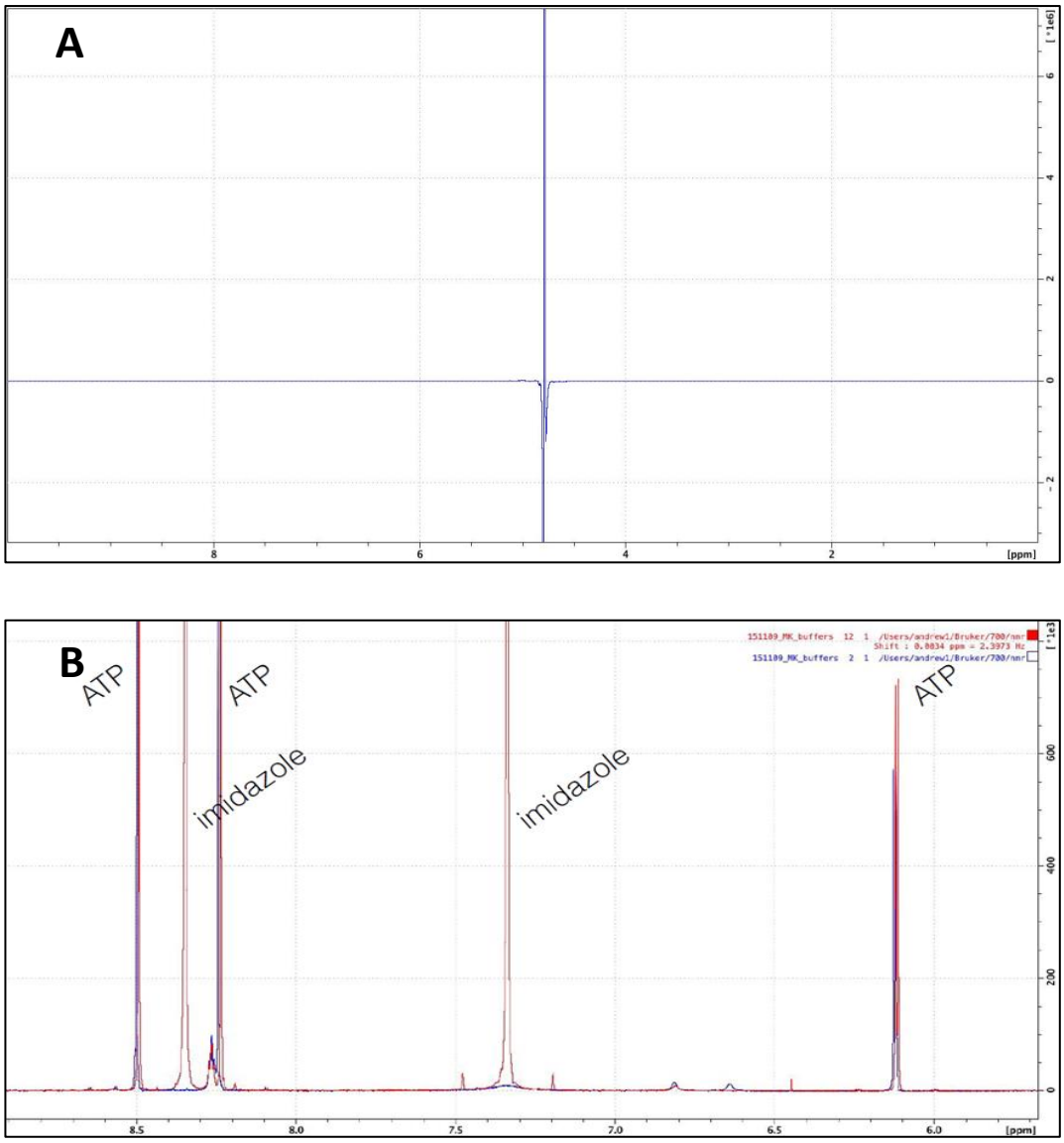


Figure 4.9. A sample portion of two ^{1}HNMR spectra.

A: The spectra corresponding to the 'blank' sample of distilled water tested. No compounds were observed other than H_2O.

B: the portion of the spectra illustrating the compounds detected in solutions B and B1. The only compounds which were found to differ were those which were already known to be different, e.g. the two large peaks corresponding to Imidazole (pictured) which were in solution B1 (red peaks) but not solution B (blue peaks).

4.3.5 Use of Glutathione (GL)

GL is a reducing agent that is a constituent part of solution B, but was absent in solution A. To assess the effect of GL on specific force a new solution with the same constituents as solution B but which excluded GL was made, called solution B2. The mean specific force measured from skinned fibres activated twice in both solutions B and B2 were not significantly different ($p > 0.05$) in all fibre types examined (Figure 4.10A). However, a significant effect of GL on the t_{50} was observed. Fibres of unclassified type and MHC I fibres activated in solution B had a significantly ($p < 0.05$) faster t_{50} than the same fibres activated in solution B2. While the same trend was observed for MHC IIA fibres, the difference was not statistically significant (Figure 4.10B).

4.3.6 KCl vs K-prop

To adjust the ionic strength, KCl was used in solution A, whereas K-prop was used in solution B. Solution A1 was made to be essentially the same as solution A except that the ionic strength was adjusted using K-prop instead of KCl, which reduced the concentration of free Cl^- ions in solution.

The specific force elicited by activating solutions A and A1 from fibres of unclassified MHC isoform and MHC I fibres, was not significantly different ($p > 0.05$). However, MHC IIA specific force was significantly lower ($p < 0.05$) in fibres contracting in solution A1, containing K-prop, compared with solution A which contained KCl (Figure 4.11A). The t_{50} of skinned fibres which contracted in solutions A and A1 was significantly ($p > 0.05$) faster in solution A1 for all fibre types examined (Figure 4.11B).

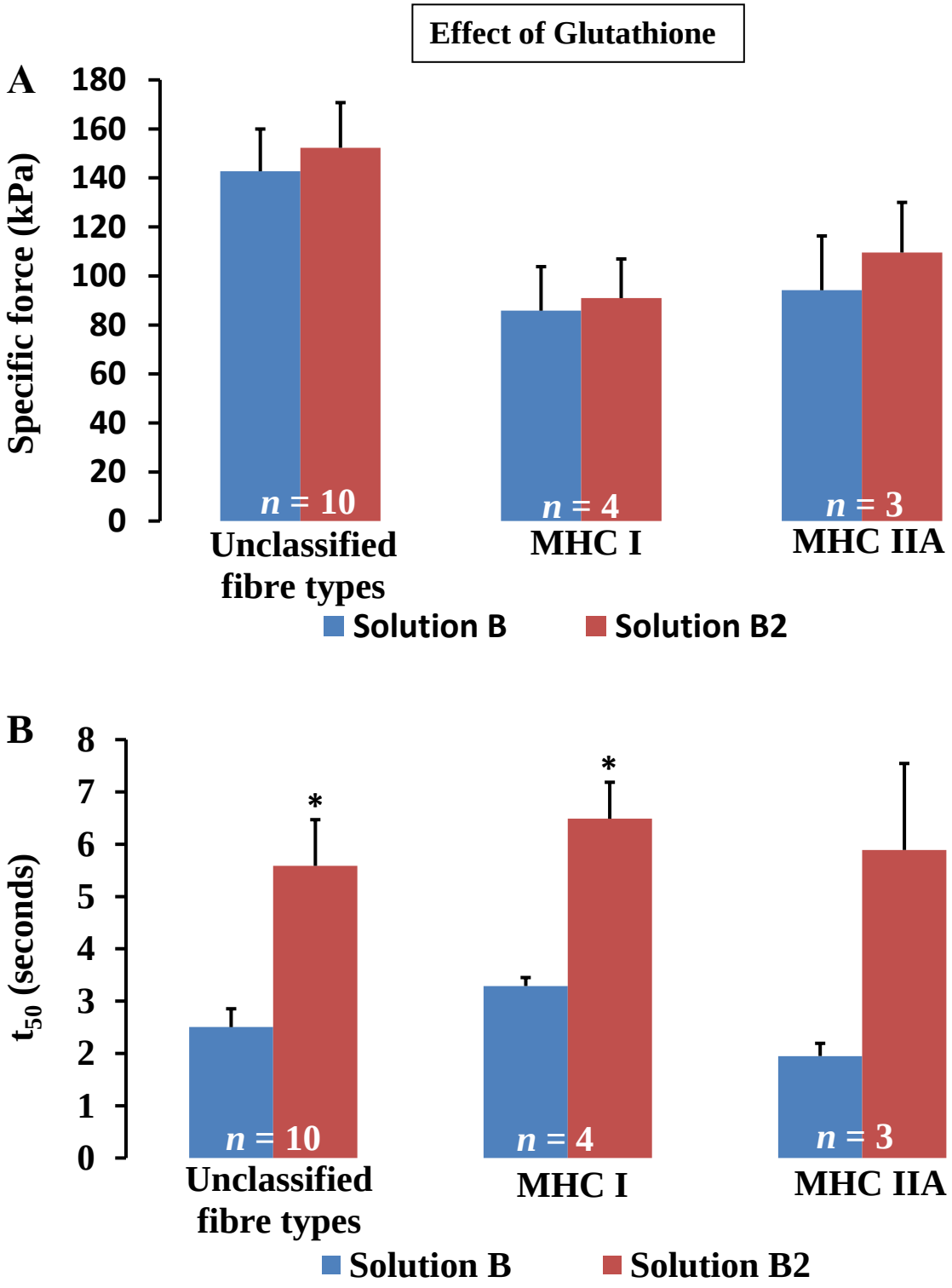


Figure 4.10. The specific force and t_{50} of skinned fibres in solution B vs solution B2. A: The mean specific force of the same individual fibres activated in solution B vs solution B2. Glutathione was excluded from solution B2. Specific force was not significantly different ($p > 0.05$) for all fibre types examined. B: The mean t_{50} from the same individual fibres activated in solution B vs B2. t_{50} was significantly ($p < 0.05$) quicker in fibres of unclassified type and in MHC I fibres which contracted in solution B, compared with solution B2 (indicated by the asterisk). Despite a trend for t_{50} to be quicker in type IIA fibres contracting in Solution B compared with solution B2, the difference was not statistically significant.

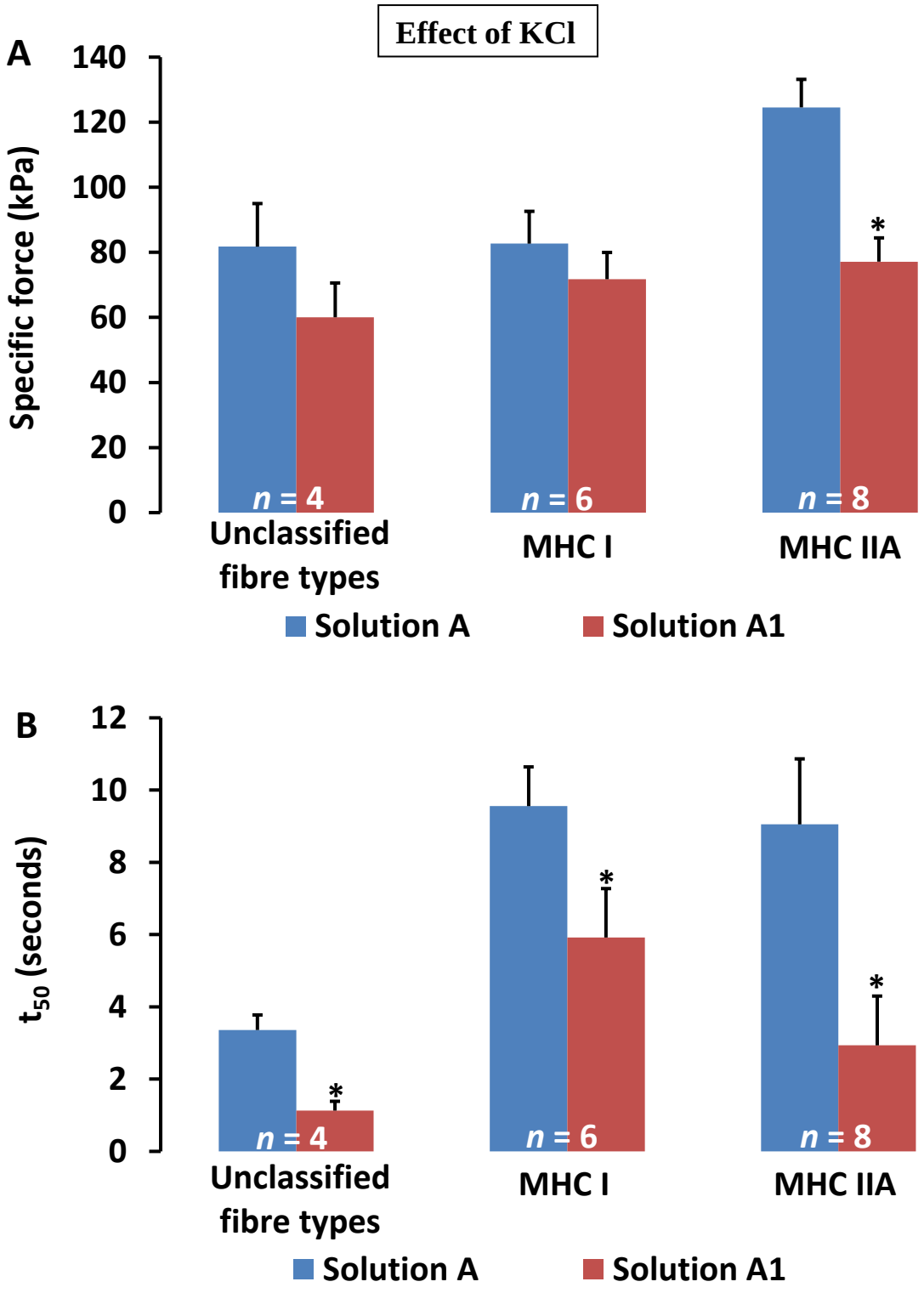


Figure 4.11. The specific force and t_{50} of skinned fibres in solution A vs solution A1. **A:** The mean specific force of the same individual fibres activated in solution A vs solution A1. K-prop was used instead of KCl to adjust the ionic strength of solution A1. MHC IIA fibre specific force was significantly ($p < 0.05$) lower in solution A1 compared with solution A. No other significant interactions were observed. **B:** The mean t_{50} from the same individual fibres activated in solution A vs A1. t_{50} was significantly ($p < 0.05$) quicker in all fibre types examined which contracted in solution A1, compared with solution A, indicated by the asterisk.

4.4 Discussion

The present study sought to investigate whether the contractile responses elicited from skinned skeletal muscle fibres were different when using different activating solutions. Each activating solution tested is used by a research group that routinely measures specific force from skinned fibres. Solution A (Larsson and Moss, 1993) is used by a large proportion of the publications studying skinned fibres from humans. Approximately 45% of publications included in the systematic review in Chapter 2 used Solution A. Solution B is used by a group which typically studies mammalian, but not human, skinned fibres (West et al., 2013). The main findings were that skinned fibres exhibited different contractile responses in each of the two activating solutions, both with regard to specific force produced and t_{50}. Furthermore, the cause of the difference in specific force elicited was identified to be the use of Imidazole in solution A, which has a depressive effect on force, and a faster t_{50} was associated with a lower free Cl^- concentration and use of Glutathione (GL), in solution B.

4.4.1 Activation protocol used

In order to differentiate between the effect of each individual solution on the contractile response from a skinned fibre the protocol employed needed to ensure that specific force measured was not affected by the order in which solutions A or B were used. Furthermore, skinned fibres can become damaged due to repeated activations, so a protocol was necessary which could indicate that a give skinned fibre remained structurally intact throughout an experiment. There was no significant difference in the specific force measured from multiple contractions of a fibre in the same solution, but there were significant differences in specific force measurements elicited between the two different solutions. This indicates that the order in which the solutions were used did not affect the results obtained and demonstrates that skinned fibre force is sensitive to the

experimental solutions used. Furthermore, these results verify that the structural integrity of a given fibre was maintained throughout an experiment, indicating that results were not influenced by damage to the skinned fibre. Therefore, the protocol utilised in the present investigation was appropriate for studying the effects of two different solutions on the contractile responses of skinned fibres.

4.4.2 Differences in specific force and time to half peak tension (t_{50}) in solutions A and B

Several differences in the contractile response of skinned fibres in solution A compared to solution B were identified and quantified. The specific force recorded from type I fibres in solution B was significantly higher ($p < 0.05$) than from the same fibres contracting in solution A. While the same trend was observed for type IIA fibres, the higher specific force recorded in solution B compared with solution A was not statistically significant. This could imply that MHC I fibre specific force was preferentially increased in solution B, compared to type IIA fibre specific force.

The t_{50} of fibres contracting in solution B was significantly faster ($p < 0.05$) compared to when they contracted in solution A, for all fibre types studied. These findings indicate that the use of different experimental solutions by different research groups is likely to be a significant contributor to the variation observed in published specific force values measured from human skinned fibres, discussed extensively in Chapter 2.

4.4.3 Implications for the inclusion of data in a given study

The present study's results indicate that an investigator's interpretation of a skinned fibre's specific force is influenced by the methodological treatment of the fibres by using a given activating solution. The activating solution used to measure specific force from skinned fibres may also affect the interpretation of a given data set and

subsequent conclusions drawn by affecting which data are included in data analysis. Given that skinned fibre specific force is greater in solution B than in solution A, some fibres activated in solution A may be excluded from data analysis on the basis that they are 'weak' when actually they have been contracting sub-optimally. For example, if a fibre's specific force data were only included if the specific force was greater than 100kPa, of the same fifty five fibres which were compared in solution A vs B, sixteen tested in solution A would be included, compared with thirty six fibres tested in solution B. This large difference in the amount of data included in subsequent analysis suggests that the same statistical analysis could lead to the formulation of different conclusions due to the amount of data studied. Furthermore, the difference in the number of fibres included in data analysis based on their specific force suggests that this would be an inappropriate inclusion criterion. Rather, the consistency of force production used in the present study is a more appropriate criterion for the inclusion of force data as 'weak' fibres, which may also occur physiologically, are not excluded from analysis.

4.4.4 Physiological significance of the difference in specific force response

Specific force was measured from the same skinned fibres in solutions A and B. Therefore, there was no difference in the force generating potential with regard to myofibrillar protein content or number of available cross-bridges in the fibres contracting in a given solution. This indicates that the higher specific force observed in solution B is due to a difference in cross-bridge behaviour of the skinned fibres. Furthermore, this finding highlights the potential for different activating solutions to be used as a means of studying cross-bridge behaviour in skinned fibres from different cohorts, such as young and elderly groups, discussed further in Chapter 5.

Solutions A (Larsson and Moss, 1993) and B (West et al., 2013) are each used by different research groups which routinely measure force from skinned single muscle fibre segments. The fact that different research groups use different activating solutions shows that standardised, optimal experimental solutions for skinned fibre experiments have not yet been developed. The higher specific force observed in solution B indicates that solution B is more efficient than solution A for recording specific force, but further experiments would be required to verify that solution B is 'optimal.'

4.4.5 The effect of chemical differences between solutions A and B on the skinned fibre contractile response.

4.4.5.1 Effect of TES on skinned fibre specific force

Solutions A and B used Imidazole or TES respectively as a pH buffer. The effect of containing different pH buffers was first tested by measuring the specific force response to different concentrations of either TES or Imidazole, illustrated in the dose-response curve in Figure 4.6. There was no significant difference in specific force measured from the same fibres tested in solutions containing TES in concentrations ranging from 20mM to 100mM, although an optimal specific force was measured at 60mM of TES. In contrast, a separate experiment demonstrated that specific force was significantly ($p < 0.05$) lower in a solution containing 0mM TES compared to solution B, which contained an optimal TES concentration (60mM). TES was developed by Good et al. (1966) as a structural analogue to Trizma buffer, to meet the previously outlined criteria of a good pH buffer for use in biological research. The beneficial effect of TES on specific force measured in the present study indicates that at least for the skinned fibre preparation, TES is an efficient buffer.

4.4.5.2 Effect of Imidazole on skinned fibre specific force

In contrast to the positive effect of using TES as a pH buffer, Imidazole in concentrations greater than 20mM had a negative effect on specific force. Specific force decreased in a linear fashion in solutions containing between 20-100mM of Imidazole (Figure 4.6), suggesting that the reduction in specific force was proportional to the Imidazole concentration in solution. No significant difference ($p > 0.05$) in specific force was observed in solution containing no Imidazole (0mM) compared to 10, 15 and 20mM of Imidazole. Therefore, there appears to be no benefit to including Imidazole as a pH buffer for skinned fibre specific force generation, in contrast to the inclusion of TES as a pH buffer, which increased specific force generation.

The Imidazole dose response curve (Figure 4.6) demonstrated that specific force was significantly lower ($p < 0.05$) in solutions containing 80mM and 100mM of Imidazole, compared to 20mM of Imidazole. This reduction in specific force could be related to the fact that the ionic strengths of the solutions containing 80mM and 100mM of Imidazole were higher, at 210 and 220mM respectively. Previous work has shown reductions in skinned fibre force in solutions of high ionic strengths of approximately 300-500mM relative to solutions of lower ionic strengths of 150-200mM (Gordon et al., 1973). This range is considerably higher than in the present study, where an increase in ionic strength of 10-20mM is unlikely to have a significant effect on skinned fibre specific force production. Indeed, Ashley and Moisescu (1977) observed that a difference in ionic strength of approximately 25mM did not affect the force-pCa relationship in barnacle muscle fibres. This implies that the reduction of specific force in solutions of higher Imidazole concentrations is primarily due to the presence of Imidazole, as opposed to the slightly higher ionic strength.

Skinned fibre specific force was significantly lower ($p < 0.05$) in a solution containing 5mM of Imidazole, reaching a similar mean value to that recorded in a solution containing 60mM of Imidazole. This is difficult to explain but based on this similarity one possibility is that an error in solution making occurred and that the concentration of Imidazole in solution was in fact approximately 50mM, not 5mM. This is supported by the fact that specific force was not significantly different ($p > 0.05$) in solutions containing between 0, 10, 15 and 20mM of Imidazole.

4.4.5.3 Concentrations of TES and Imidazole used

Interestingly, solution B contains 60mM of TES, which in the present study was found to be the optimal concentration for skinned fibre specific force generation. Also, the majority of studies in the human skinned skeletal muscle fibre literature use an activating solution containing 20mM of Imidazole, which the present study identified as being in the optimal range for specific force production, in solutions using Imidazole as a pH buffer (Figure 4.6). These two observations suggest that at some point research was conducted to determine the appropriate concentrations of compounds in experimental solutions used to study the contractile response of skinned fibres, the knowledge from which has been utilised by research groups studying muscle physiology. However, based on extensive literature searching and communication with experts in muscle physiology (Emeritus Professor George Stephenson, Professor Jon Kentish), it appears that the research which may have identified optimal concentrations of TES, Imidazole and other compounds investigated in the present study, has not been published. One possible reason for the use of 20mM of Imidazole in the activating solutions used, is that Ogawa (1968) reported that the Ca-EGTA binding constant increased as Imidazole decreased below 20mM, which could have implications for the force-pCa relation in skinned fibres.

4.4.5.4 TES vs Imidazole

In order to determine whether TES or Imidazole was more effective at eliciting specific force from skinned fibres at their respective optimal concentrations, the two compounds were directly compared against each other. Specific force was measured from skinned fibres contracting in solution B (containing 60mM of TES) and solution B1 (containing 20mM of Imidazole) which were almost identical in chemical composition but for the use of the different pH buffers. A concentration of 20mM of Imidazole was selected for solution B1 as the present study found this to be in the optimal range for specific force production, which would also enable a comparison with the majority of the human skinned fibre literature, which uses the same concentration. Specific force was consistently (Figure 4.8) and significantly greater ($p < 0.05$) (Figure 4.7) in solution B compared with solution B1, for MHC IIA, IIA-X fibres and fibres of unclassified fibre type. MHC I fibres exhibited the same trend but only two MHC I fibres were identified, so a statistical comparison was not possible.

The consistency of the difference in specific force elicited by the two solutions suggests that the present investigation succeeded in isolating the effect of a single chemical compound, in this case Imidazole, on the skinned fibre contractile response. Therefore, the lower specific force observed in solution A in the present study is at least in part due to the use of Imidazole as a pH buffer. Furthermore, this finding clearly suggests that the majority of publications are taking sub-optimal force measurements from human skinned fibres by using Imidazole as a pH buffer instead of TES. Sub-optimally activating skinned fibres may affect the conclusions drawn from a given study either by affecting the relationship between type I and type IIA fibres, or by affecting which data are included for statistical analysis, as has been previously discussed.

4.4.5.5 Potential mechanism of action of Imidazole

A potential mechanism of the depressive effect of Imidazole on skinned fibre force appears to be how Imidazole influences ionic strength. The ionic strength of the solutions used to directly compare the effect of either TES or Imidazole on force was the same (200mM), but the amount of K-prop required to maintain the ionic strength in either solution differed. Indeed, in order to accommodate Imidazole concentrations of 80mM and 100mM the ionic strength had to be raised to 210mM and 220mM respectively. In contrast, 80 and 100mM of TES were easily accommodated in solution without alterations in ionic strength. This suggests that Imidazole exerts a different effect on the ionic strength of a solution compared with TES. The impact of Imidazole and TES on the ionic strength of solutions B and B1 could not be assessed using the ^{1}HNMR experiments employed in the present study. Also, the ionic strength of the solutions was calculated, not measured, in the present study, which could introduce some error into the actual ionic strengths of each activating solution.

Given that the ionic strength is a measure of the concentration of free ions in solution, the effect of Imidazole may simply be to increase the availability of ions such as Mg^{2+} which may suppress force generation. Increases in the millimolar range of Mg^{2+} had a significant inverse effect on sub-maximum Ca^{2+} activated force production in frog muscle. This was associated with decreased binding of Ca^{2+} to myofibrils at a given Ca^{2+} concentration, and was attributed to Mg^{2+} competing with Ca^{2+} for binding sites on the myofilaments (Donaldson & Kerrick, 1975). Indeed, Ashley & Moisescu (1977) showed that increasing [Mg^{2+}] caused a shift in the tension-pCa curve such that a higher [Ca^{2+}] was required for a given amount of force. This was also attributed to competitive inhibition between Mg^{2+} and Ca^{2+} for the functional unit. The ^{1}H NMR experiments employed in the present study did not detect any compounds in solutions B and B1 which

were not already known to be there based on the chemical composition of the two solutions. However, metal ions such as Mg^{2+} or Ca^{2+} could not be visualised using ^{1}HNMR as they do not contain H^{+} atoms, so the competing action of these two ions cannot be ruled out based on the ^{1}HNMR data.

The ionic strength of solutions B (containing 60mM of TES) and B1 (containing 20mM of Imidazole) were the same. Therefore, the negative effects of increased ionic strength on skinned fibre force would not have caused the observations made in the present study, but may provide some indication as to the effect of Imidazole on cross-bridge behaviour, if Imidazole does indeed affect ionic strength. P_0 of rabbit psoas fibres measured at 3-5°C was reduced by increasing the ionic strength of the activating solution between 120-520mM. The decline in P_0 was associated with a reduced skinned fibre stiffness. Interestingly, stiffness increased relative to force at high ionic strengths, suggesting that fewer cross-bridges are formed and force produced by each cross-bridge is lower at high ionic strengths (Iwamoto, 2000). Consistent with these findings, rabbit psoas fibre P_0 measured at 20°C was reduced when the activating solution ionic strength was increased between 150-300mM, which was also associated with reduced stiffness (Kawai et al., 1990). Furthermore, lowering ionic strength increased P_0 in rabbit psoas fibres, which was associated with increased fibre stiffness (Sugi et al., 2013). Therefore, assuming Imidazole exerts a different effect on ionic strength compared to TES, a potential explanation of Imidazole's negative effect on specific force is reduced fibre stiffness.

In contrast to the aforementioned findings from studies conducted at sub-physiological temperatures, West et al. (2005) studied the effect of ionic strength on P_0 in dog fish fibres contracting at temperatures (12°C) which are physiological for that species. Increased ionic strength did not negatively affect P_0 in dog fish fibres contracting

at physiological temperatures, although the rate of force development was reduced. Therefore, changes in ionic strength may not affect P_0 *in vivo*, since a given muscle would be contracting at a physiological temperature. However, the experiments in the present thesis and in the majority of the human skinned fibre literature are conducted at sub-physiological temperatures (12-15°C), indicating that maintaining an appropriate ionic strength is relevant for P_0 measurements.

Given that Imidazole is a pH buffer, a simple explanation for the negative effect of Imidazole on skinned fibre specific force, is that Imidazole does not adequately maintain activating solution pH. Indeed low pH has been associated with reduced skeletal muscle fibre force production. Low intracellular pH (pH 5.97) caused a reduction in P_0 from intact frog fibres compared to neutral (pH 7.24) values (Curtin et al., 1988). In skinned rat fibres from superficial portion of the Vastus Lateralis, lower pH (pH 6.2) reduced P_0, which was attributed to a reduction in fibre stiffness (Metzger and Moss, 1989). The negative effect of lowered pH on force production from intact mouse flexor brevis fibres was shown to be reduced at higher experimental temperatures (32°C compared to 12°C and 22°C), although the effect was still significant (Westerblad et al., 1997).

The pH of the activating solutions in the present study was carefully adjusted to pH 7.1 when the solutions were made. However, pH could not be measured during an experiment due to the small volume (approximately 60µl) of activating solution used for each skinned fibre contraction. The activating solution temperature was controlled and monitored throughout a given experiment, indicating that any potential changes in pH would not be due to changes in the temperature of the solution. Furthermore, as previously mentioned, skinned fibre specific force data were included based on the consistency force production, so any inconsistencies in specific force due to changes in activating solution

pH would have been excluded. Therefore, that the effect of Imidazole is due to a change in the activating solution pH in the present study in unlikely.

4.4.6 The rationale for using Imidazole as a pH buffer in activating solutions

Some evidence exists, which although limited, may have been used as the rationale for utilising Imidazole as a standard pH buffer in activating solutions used to study skinned skeletal muscle fibres, prior to the technique becoming widely used in human research from the 1990s onwards. The following section assesses some of the reasons why Imidazole has been used in activating solutions, despite the observations made in the present study that Imidazole inhibits skinned fibre specific force.

4.4.6.1 pH buffering in vivo: Imidazole containing dipeptides

Smith (1938) studied skeletal muscle in rigor, as this model provided a simpler buffering system to analyse than that present in living muscle, and observed that the Imidazole containing dipeptides Carnosine and Anserine, accounted for approximately 35% of the buffering of mammalian skeletal muscle, at least in rigor. Smith (1938) noted that Carnosine and Anserine were suitable for buffering between pH 6.5-7.5 due to their pK characteristics. Davey (1960) commented that the buffering actions of Carnosine and Anserine would prevent dangerous falls in pH due to H^+ ion production as a result of anaerobic glycolysis during and following strenuous activity. Indeed, in humans a higher Carnosine content has been quantified in MHC II fibres compared to MHC I fibres, evidencing that Imidazole containing dipeptides are likely to contribute to H^+ buffering in human skeletal muscle (C. Harris et al., 1998). Furthermore, increases in muscle

Carnosine content due to β-alanine supplementation were shown to attenuate fatigue during repeated dynamic contractions of the quadriceps muscle (Derave et al., 2007).

The aforementioned findings support that Imidazole containing dipeptides such as Carnosine have a role in H^+ buffering in human skeletal muscle. However, an important point is that Carnosine is not used as a pH buffer in activating solutions, rather, the Imidazole ring of the Carnosine molecule is used (Figure 4.12). Therefore, while Imidazole may be utilised as a structural analogue to dipeptides which contribute to H^+ buffering *in vivo*, either the effect of the Imidzole ring alone does not provide as efficient a H^+ buffering capacity, or the Imidazole ring may simply be inappropriate for the skinned fibre preparation.

4.4.6.2 Experimental evidence which may have supported the use of Imidazole in activating solutions used to study skinned fibres

Bowen (1965) demonstrated that shortening of glycerinated bundles of rabbit psoas fibres was facilitated by the inclusion of the Imidazole containing dipeptides, Histidine and Carnosine, in solution. Murphy and Koss (1968) later identified both Imidazole and TES as appropriate pH buffers for maintaining pH between 6-7, where ATPase activity was optimal. Furthermore, Imidazole was described as an economical pH buffer, indicating that perhaps the initial and continued use of Imidazole was due to financial reasons. This study was conducted on homogenised rabbit muscle, so the impact of pH buffers on skeletal muscle mechanical properties could not be examined.

Apart from regulating pH in the desirable, neutral range for skinned fibre experiments, Imidazole was found to not affect the ATPase activity of contractile proteins (Kerrick & Donaldson, 1972). Furthermore, Donaldson & Kerrick (1975) state that Imidazole did not seem to affect the tension-pCa relationship in skinned frog skeletal

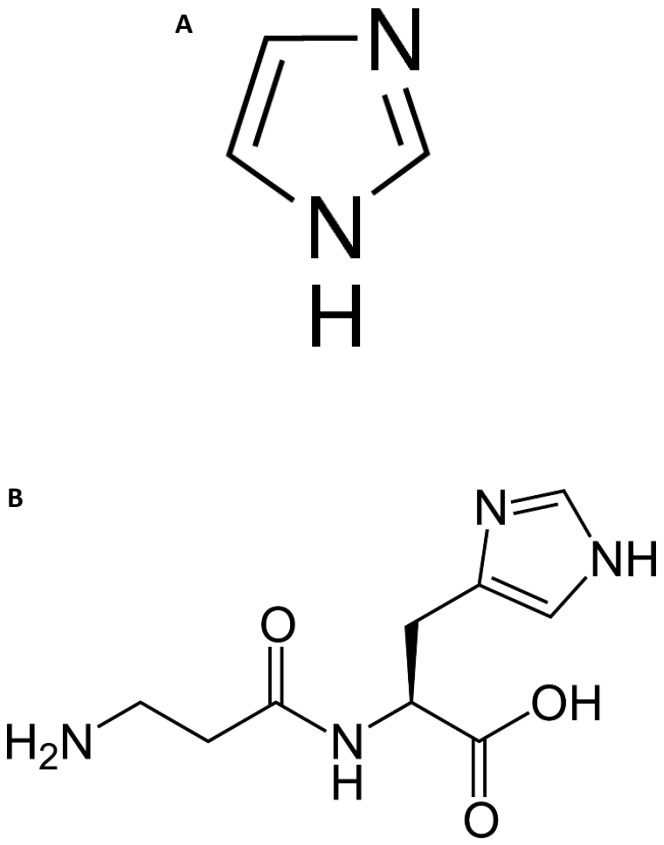


Figure 4.12. The commercially available Imidazole ring, and an example of that which is present *in vivo*.
A: The Imidazole ring is widely utilised as a pH buffer in activating solutions used to study skinned, skeletal muscle fibres. **B:** Carnosine, the Imidazole containing dipeptide observed *in vivo* in human skeletal muscle. While the Imidazole ring may provide a structural analogue to Carnosine in a given activating solution, the effect on skinned fibre specific force may not be the same as that of *in vivo* buffering constituents such as Carnosine.

muscle fibres. However, data to support this statement are not displayed and the effect of Imidazole on absolute force produced was not examined.

Studying skinned frog semitendinosus fibres Fabiato and Fabiato (1978) used Imidazole as a pH buffer to examine the effect of pH 7.4, 7.0. 6.6 and 6.2 on force generation. Interestingly, they also did experiments at pH 7.4 using Tris and or Bis-(2-hydroxyethyl)amino)-tris(hydroxymethyl)methane and observed that the change of pH buffer did not affect force at a given pH or pCa. This potentially provides a rationale for different research groups using Imidazole, under the incorrect assumption that compared with other buffers Imidazole does not negatively affect skinned fibre force production.

As previously mentioned, Imidazole has an appropriate pKa (7.09 at 25°C) for maintaining the desirable pH in solutions used to study skinned fibres. However, this is nullified by the fact that highly recommended pH buffers such as TES have pKa values (7.4 at 25°C) in the same range (Murphy and Koss, 1968). Two different pH buffers may both have desirable buffering capacities but may not be equally effective in the same experimental preparation. Therefore, Good et al. (1966) recommend that extensive testing is carried out in order to select an appropriate pH buffer, as the side effects of a pH buffer are independent to their buffering capacity in a given pH range, as has been shown in the present study.

Finally, several publications which were key (Ashley and Moisescu, 1977, Moisescu, 1976, Moisescu and Thieleczek, 1978) in the development of solutions used to study skinned skeletal muscle fibres use TES as a pH buffer. TES was preferred to Imidazole due to the latter having a pK sensitive to temperature, being unstable in solution in the presence of light and because the protonated form of Imidazole changes the sensitivity to Ca^{2+} of skinned fibres (personal correspondence with George Stephenson).

Therefore, the use of Imidazole in experimental solutions used to study skinned fibres is strange, since TES was preferred in the solutions used in some highly cited research. As this work (Ashley and Moisescu, 1977, Moisescu, 1976, Moisescu and Thieleczek, 1978) was conducted in animals, perhaps research groups working on human muscle followed a different practice, or perhaps because the main emphasis was not on the pH buffer this aspect of the experimental solutions was overlooked in future research.

4.4.7 Time to half peak tension (t_{50}) in solutions A and B

The t_{50} was significantly longer in solution A compared with solution B (Figure 4.4). A potential mechanism examined was the role of diffusion, by correlating the t_{50} with skinned fibre CSA. If diffusion was a limiting factor then t_{50} should be greater in larger fibres. However, no correlation was observed in either solution A or B, indicating that diffusion does not limit the t_{50} in skinned fibres irrespective of fibre type. This is in agreement with previous work on skinned fibres from rat hindlimb (EDL and Soleus) muscle where force equilibrated faster at higher temperatures, showing that the physical process of Ca^{2+} diffusion is not rate limiting to force development (Stephenson & Williams, 1981). Furthermore, Ferenczi et al (1984) found no appreciable correlation between V_{max} and CSA, indicating that diffusion of substrates and their subsequent concentration in the core of skinned fibres is not different between fibres of small or large diameter.

4.4.7.1 Use of Glutathione

Glutathione (GL) is a reducing agent and is not a common feature of activating solutions typically used to study human skinned fibres. The reason for including GL in

solution B is likely to be to preserve enzyme function or prevent reactive oxygen species induced damage during an experiment. Indeed, GL was found to attenuate a five-fold reduction in force produced by mechanically skinned, rat EDL fibres, caused by hydrogen peroxide (Murphy et al., 2008). The results from the present study indicate that GL is not necessary to maintain skinned fibre force production in the absence of reactive oxygen species, although no negative effect of GL was observed.

Solution B contained GL, whereas solution A did not. Excluding GL in solution B2 significantly increased the t_{50} compared with the contractile response elicited by solution B, irrespective of fibre type (Figure 4.10B). Assuming that t_{50} is an indirect measure of the rate of force development, this finding shows that the effect of GL is to increase the rate of force development in skinned fibres. Indeed, a similar observation was made by Posterino and Lamb (1996), who reported no effect of GL on mechanically skinned rat EDL fibre force, but the duration of the force response was reduced. This observation was attributed to a small, GL induced reduction in skinned fibre Ca^{2+} sensitivity. The difference in t_{50} between solutions B and B2 is not as great as between solutions A and B, suggesting that GL only partly accounts for the faster t_{50} observed in solution B.

4.4.7.2 KCl vs K-prop

Solution A1 was essentially the same as solution A but for the use of K-prop instead of KCl to adjust the ionic strength to 200mM. Since K-prop does not contain chloride, the use of K-prop instead of KCl was assumed to reduce the concentration of free Cl^- ions in solution A1. Therefore, the significantly faster t_{50} ($p < 0.05$) observed in fibres of all fibre types in solution A1 compared with solution A (Figure 4.11B), can be attributed to the effect of a lower concentration of Cl^- ions on the contractile response. The difference in t_{50} between solutions A1 and A was similar to that between solutions A

and B, suggesting that the primary mechanism causing the difference in t_{50} between solutions A and B is the greater [Cl^-] in solution A compared with solution B.

Stephenson and Podolsky (1977) observed that Cl^- ions diffused uniformly into skinned frog muscle fibres, suggesting that the skinned fibres in the present study would have been permeated with Cl^- ions, particularly in solution A which contained a higher Cl^- concentration. Furthermore, intracellular concentrations of Cl^- increase in proportion to the tonicity of a given solution, tonicity representing the solutes that cannot cross the sarcolemma, which exert osmotic pressure (Gordon and Godt, 1970). Given that the sarcolemma of skinned fibres has been permeabilised, the solution tonicity is unlikely to affect the Cl^- ions entering a given fibre. Therefore, the intracellular Cl^- concentration in the skinned fibre preparation is likely due to the Cl^- concentration in the activating solution. The presence of increasing Cl^- ion concentrations was found to affect the rate at which actin and myosin dissociate (GEEVES and GOLDMANN, 1990). However, this was observed using fluorometric titration of pyrene-labelled actin, where actin and myosin are dissociated from the myofilaments, so whether Cl^- ions affect the dissociation of actin and myosin during skinned fibre contraction is difficult to determine.

The rate of force development in skinned fibres of rat EDL and Soleus muscle was shown to depend strongly upon the ionised Ca^{2+} concentration and experimental temperature. Given that the temperature of the experiments in the present investigation was maintained at 15^0C, Cl^- may act via a mechanism which affects the free ionised Ca^{2+} concentration available to bind to the myofilaments. As the fibres in the present study are skinned using glycerol, Cl^- could potentially affect the Ca^{2+} released from the SR.

Specific force was found to be similar in fibres of unclassified type and type I fibres tested in solutions A and A1, but type IIA fibre specific force was found to be significantly lower ($p < 0.05$) in solution A1 compared with solution A (Figure 4.11A).

That a reduction in Cl^- ions should cause a reduction in type IIA fibre specific force is difficult to explain. The use of KCl to adjust ionic strength was shown to have a negative effect on force produced from skinned frog fibres, compared to the use of K-prop, even though the ionic strength of the two solutions used was the same (Gordon et al., 1973). Therefore, the anion used to adjust ionic strength can affect skinned fibre force generation. However, the effect observed was the opposite to that reported in the present study, i.e. Gordon et al. (1973) reported a decreased skinned fibre specific force due to the use of KCl, whereas an increase in type IIA fibre specific force was observed in the present study as a result of using KCl instead of K-prop to adjust ionic strength.

A practical consideration to take into account is that solution A1 was made using a custom written Matlab programme which was designed to make solution B. Therefore, solution A1 was created to mimic solution A as closely as possible, but using the same chemicals constituting solution B was necessary due to the technical limitations of the solution composition computer programme. The implication of this technical limitation is that the sole difference between solutions A and A1 was not exclusively the concentration of Cl^-. Therefore, the reduction in specific force observed in MHC IIA fibres contracting in solution A1 cannot be solely attributed to the impact of a reduction in Cl^- concentration, although this is the primary difference between solutions A and A1.

4.4.8 Summary and conclusions

The main findings in the present chapter were that the contractile response of the same skinned fibres was markedly different depending on the activating solution used. This strongly suggests that the variance in skinned fibre specific force reported in the literature is contributed to by the lack of consistency in the chemical composition of the activating solutions used across different studies. Furthermore, Imidazole, a pH buffer,

was found to be the primary compound affecting force production and had a negative effect on skinned fibre specific force. A lower concentration of Cl- ions and the inclusion of Glutathione was found to be associated with a faster time to half peak tension, implying that the rate of force development could be affected by these two chemicals, although this would need to be verified with further experiments. The fact that the same fibres, which by definition have the same force generating potential, produce different amounts of force in solutions A and B, indicates that the use of two different activating solutions can be used as a tool to reveal differences in cross-bridge behaviour. While the present study has shown that solution B induces a stronger and faster contraction from skinned fibres, determining whether solution B is 'optimal' requires further research.

5 The impact of physical activity on the development of age-related specific force loss in human skinned fibres.

5.1 Introduction

Specific force loss at the whole muscle level in elderly humans was first observed by Men et al. (1985), who reported that quadriceps P_0 was 39% weaker in elderly compared to young men, but quadriceps CSA measured using ultrasound was only 25% smaller. As discussed in section 0, a host of methodological factors need to be taken into account in order to obtain a reliable, whole muscle specific force measurement. Studies which have accounted for all, or at least the majority of these factors have confirmed that specific force loss can occur at the whole muscle level in healthy, elderly individuals (Klein et al., 2001, Macaluso et al., 2002, Morse et al., 2005).

Measuring specific force *in vivo* cannot provide information as to whether the mechanism of age-related specific force loss is due to alterations or deterioration in the function of the force generating myofilaments in individual skeletal muscle fibres. Therefore, the skinned fibre preparation has been used to assess whether age-related specific force loss is due to changes in the contractile properties of the myofilaments, as a result of advancing age (D'Antona et al., 2007, Frontera et al., 2003, Frontera et al., 2000a, Hvid et al., 2011, Hvid et al., 2013, Korhonen et al., 2006, Miller et al., 2014, Ochala et al., 2007, Power et al., 2016, Reid et al., 2012, Reid et al., 2014, Venturelli et al., 2015, Larsson et al., 1997, Yu et al., 2007, D'Antona et al., 2003, Grosicki et al., 2016, Callahan et al., 2014).

As described in section 1.7.2, the observation that specific force loss occurs in skinned Vastus Lateralis fibres from sedentary elderly, compared to young people has been reported inconsistently. Nevertheless, trends in specific force between young and

elderly cohorts have been mirrored by differences, or lack thereof, in myofibrillar protein content (section 1.7.7.1). This indicates that the mechanism underpinning a given trend in reported skinned fibre specific force, with regard to myofibrillar protein content, is consistent. Indeed, D'Antona et al. (2003) demonstrated that skinned fibre specific force has a linear relationship with skinned fibre myosin content, and that lower specific force in elderly cohorts is associated with lower myosin content. These two measurements were taken from separate sets of fibres (Figure 1.8). Consistent in principle with these findings, elderly and young cohorts exhibited similar skinned fibre specific force, and analogously had a similar myofibrillar protein content, although this was measured in homogenate muscle samples (Trappe et al., 2003).

The equivocal results regarding age related specific force loss at the skinned fibre level have also been attributed to the varying daily physical activity levels of participants studied, and reports suggest that skinned fibre specific force loss is ameliorated by physical activity (D'Antona et al., 2007). Indeed, studying physically active elderly cohorts such as master athletes has been proposed as a model of biological ageing free from the negative effects on health of a sedentary lifestyle (Pollock et al., 2015, Lazarus and Harridge, 2010).

In addition, methodological differences between studies such as the different activating solutions used to elicit maximal isometric contractions from skinned fibres are a likely source of the variability in published results. As discussed extensively in Chapter 4, the same skinned fibres produced a markedly different contractile response, revealing differences in cross-bridge behaviour, depending on the activating solution used to elicit contractions. The use of different activating solutions may therefore provide a potential means to further investigate the mechanisms of age-related specific force loss. Furthermore, techniques which provide information as to the structure of the myofibrillar

proteins during contraction, such as low-angle X-ray diffraction, may also help reveal the cause of any observed age-related specific force loss.

Therefore, the aims of the present chapter were to:

1. Assess the impact of physical activity on the development of specific force loss at the skinned fibre level, using master cyclists as a model of biological ageing, compared to comparatively frail hip fracture patients and a young, healthy cohort.
2. Evaluate whether the use of two different activating solutions (solutions A and B) would yield two different conclusions from the same skinned fibres, as to the occurrence of age-related specific force loss.
3. Measure myosin content of the same skinned fibres from which specific force has been measured, to determine whether any specific force differences observed between groups could be attributed to differences in skinned fibre myosin content.
4. Use low-angle X-ray diffraction to obtain preliminary data regarding myofibrillar structure during contraction in skinned fibres from young and elderly cohorts, to better understand the cause of any observed differences in specific force.

5.2 Methods

5.2.1 Subjects

Three mixed sex cohorts were recruited to participate in the present study (section 3.1). Five young participants (mean age: 25.8 ± 4.7 years), five master cyclists (mean age: 74.8 ± 3 years) and five patients who underwent dynamic hip screw insertion surgery (mean age: 74.6 ± 9 years) provided informed consent to participate in the present investigation. The study was approved by the King's College London ethics committee and ran in accordance with the declaration of Helsinki (1964). The age and gender of the participants are provided in Table 5.1.

Elderly patients undergoing dynamic hip screw insertion surgery aged 55 years or older were identified by Mr Onur Berber or Mr Marc George, who were members of their healthcare team. Patients underwent mental health screening to ensure that they were able to provide informed consent prior to surgery. The patients also completed a basic physical health questionnaire and were considered eligible if they did not suffer from any neuromuscular disease. The hip fracture patients were recruited on the basis that they were likely to be frail individuals, and due to their age they sometimes had underlying health problems, thereby making up a heterogeneous elderly group.

Physically active elderly individuals who were amateur, non-elite cyclists aged between 55-79 years old were recruited for a cross-sectional study evaluating the relationship between physiological function and age (Pollock et al., 2015). Based on a health questionnaire, participants were excluded if they smoked or consumed alcohol excessively, had known hypertension or any known cardiovascular, musculoskeletal or neurological conditions, or if they were on any medication. Participants were considered healthy if they met the criteria outlined by Greig et al. (1994). Males and females were included if they were able to cycle 100km in under 6.5 hours or 60km in under 5.5 hours,

respectively. Participants had to have completed the aforementioned distance within the specified time on two occasions in the three weeks prior to testing, as shown by their training logs or official timing cards. Therefore, these participants formed a homogeneous group of physically active elderly individuals, hereby referred to as 'master cyclists.'

5.2.2 Biopsy and preparation of muscle samples for experiments

A muscle biopsy was taken from the Vastus Lateralis using a Bergstrom biopsy needle with suction (Bergstrom, 1962), and fibres were prepared as previously described (section 3.3). The muscle sample was dissected into bundles of approximately fifty to one hundred fibres which were chemically skinned, as described in section 3.4. All bundles were processed for long-term storage (section 3.5) and subsequently de-sucrosed before being prepared for experimentation (section 3.6), to ensure that all bundles had undergone the same treatment prior to force measurements being taken. Once individual skinned fibres had been dissected from a bundle, t-clipped and mounted to the experimental set up (section 3.7) sarcomere length was set to an optimal length of 2.75µm and the dimensions of a given skinned fibre were measured (section 3.8). Skinned fibre CSA was calculated assuming a cylindrical fibre shape, without adjusting for fibre swelling.

5.2.3 Force measurements from chemically skinned skeletal muscle fibres

As described in section 3.9, skinned fibres were transferred from a relaxing (pCa 9.0) to a pre-activating solution, followed by an activating solution (pCa 4.5). The fibres contracted until a plateau in force signal was reached, at which point they were transferred back into relaxing solution. Peak isometric force (P_0) was classified as the baseline force signal of the fibre in the activating solution subtracted from the maximum force signal

recorded (section 3.9). Specific force was calculated as the skinned fibre P_0 normalised to CSA.

The two different activating solutions described extensively in Chapter 4 were used to measure force from the same skinned fibres obtained from each participant studied. The same batch of solution A and solution B were used for all skinned fibre specific force measurements in the present study. Therefore, the five contraction protocol which was described in section 4.2.5 was utilised in order to ensure that the force results obtained were not affected by any structural damage to skinned fibres, or the order in which the two different activating solutions were used. Fibres were visually inspected prior to a given experiment, and fibres which had small tears or nicks were excluded. The same inclusion criteria described in section 4.2.5 was used in the present study, i.e. data were included for analysis if there was a coefficient of variation of < 28% in specific force within a pair of contractions, measured in the same activating solution.

5.2.4 Modification made to the skinned fibre test system

A technical modification was made to the solution changing system described in section 3.7, in order to reduce the signal artefact caused by the upwards and downwards movement of the metal trough, previously described in section 3.10. A coverslip was placed over the top of the metal trough and the relevant experimental solutions were contained within a ring of hydrophobic ink. Thus, in order to move a skinned fibre from solution to solution only sideways movement was required. This technical alteration to the solution changing system reduced the signal artefact caused by the upwards and downwards movement of the metal trough (Figure 5.1) and also reduced the time required for the fibre to be transferred to a different solution.

Following the previously described technical modification to the solution changing system, the Matlab programme used to calculate the skinned fibre baseline force, described in section 3.10, also needed to be modified. The reduction in the signal artefact due to the technical modification enabled clear baseline and peak force signals to be observed, as can be visualised in (Figure 5.1). Therefore the Matlab code was simplified so that the baseline and peak force signals were both calculated independently as the average signal between two manually selected points. The baseline signal could then be subtracted from the peak force signal to calculate P_0.

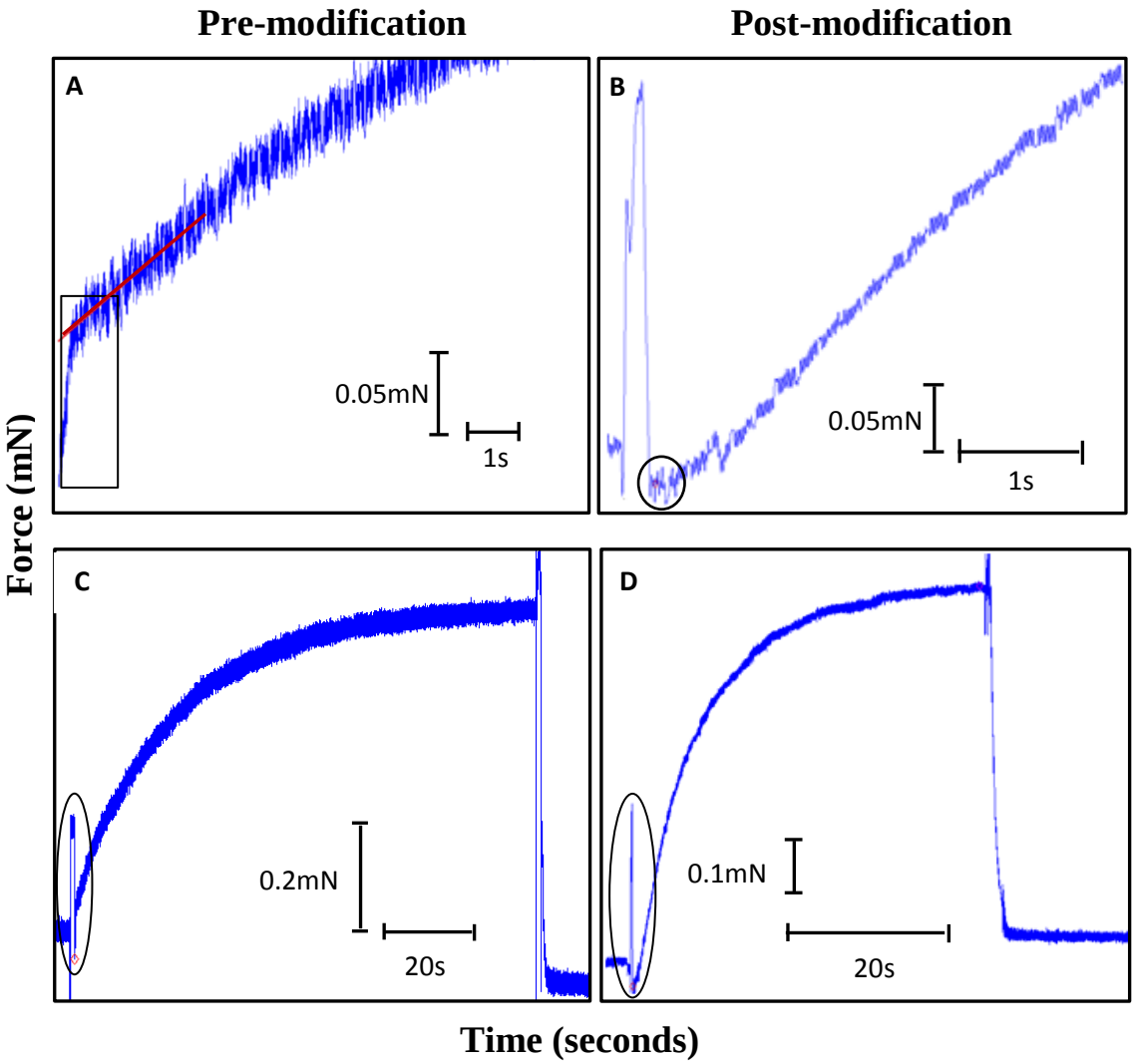


Figure 5.1. Representative images of a skinned fibre force trace before (A & C) and after (B & D) the technical modification made to the solution changing system.

A and C: A zoomed in image of the signal artefact which concealed the baseline signal (A), prior to the technical modification (described in section 5.2.4). The whole force trace is pictured in C and the zoomed in section of the signal is circled.

B and D: A zoomed in image of the flat baseline signal which could be measured (B), following the technical modification (described in 5.2.4) to the solution changing system. The whole force trace is pictured in D and the zoomed in section of signal is circled. Time, on the horizontal axis was measured in seconds, and force was measured in mN.

5.2.5 SDS-PAGE

SDS PAGE was carried out in order to identify the MHC isoform expression of individual skinned fibres (section 3.11). The myosin content of individual skinned fibres was determined by quantifying the optical density of their MHC bands on SDS PAGE gels in relation to bands corresponding to myosin standards of known myosin content (section 3.12). Myosin content and specific force from the same fibres were correlated and the relationship between these two variables was assessed in each experimental group studied. Myosin content data is available from fourteen of the fifteen participants studied.

In order to ensure that the calculated amounts of myosin in each MHC band were reliable, the linear range of the myosin standard's optical density was identified (section 3.12). Furthermore, whether the sample buffer used to de-nature and extract myosin from skinned fibres had a homogeneous effect within the same sample, was tested. This was carried out using two skinned fibre samples from a young individual, by applying two different loads of each sample to the same gel. The brightness area products (BAPs) of the subsequent MHC bands were expressed per µl of sample loaded, in order to assess whether the same relative BAP was present in the two different loads of the same sample (Figure 5.7).

A new batch of Laemmli sample buffer (LSB) was made half-way through the present study and used to prepare skinned fibre samples for SDS PAGE gels. Therefore, whether the new batch of LSB had the same effect on the myosin standard was tested by processing the myosin standard in the old and the new batch of LSB, and quantifying two myosin standard curves from the same gel (Figure 5.8A). Furthermore, whether each batch of LSB had a homogeneous effect across different samples was tested. Four skinned fibres were cut into halves of equal length which were t-clipped, mounted to the skinned fibre test set up, and their dimensions were measured. Each half of a given skinned fibre

was processed in the same LSB. The samples were prepared using either the old or the new batch of LSB, processed for SDS PAGE experiments and their MHC bands were analysed. The calculated myosin content normalised to fibre volume for each half is presented in Figure 5.8B.

5.2.6 X-ray diffraction

Low-angle X-ray diffraction experiments were carried out at the Spring-8 synchrotron facility in Japan, to garner information as to the structure of the myosin filament heads bound to actin during a skinned fibre isometric contraction. These experiments were organised by Dr Julien Ochala, in collaboration with Dr Hiroyuki Iwamoto. Bundles of skinned fibres to be prepared for low-angle X-ray diffraction experiments were de-sucrosed (section 3.5) and single fibres were dissected in relaxing solution. Individual fibres were clamped between two halves of a 3mm wide gold mesh, typically used for electron microscopy, which had been attached to a precision-machined ceramic chip (Figure 5.2A). Each pair of gold meshes held approximately thirty fibres, termed a 'set.' The sets of fibres were designed to fit the specimen chamber (Figure 5.2B) at the BL45XU beam line, where the samples were studied. Sets were mounted with skinned fibres from all participants in the young and master cyclist groups. However, only one hip fracture patient muscle sample had been obtained at the time of the X-ray diffraction experiments, so fewer sets were mounted for the hip fracture patient group and subsequently the data collected are pilot data.

Due to the nature of X-ray diffraction experiments, large numbers of skinned fibres are necessary as radiation damage during the experiment causes a substantial number of skinned fibres to break. Therefore, sets of fibres were prepared for each experimental group, as opposed for each participant studied, simply because preparing

the high number of samples required to obtain visible diffraction patterns for individual subjects would be practically impossible.

The majority of the sets containing skinned fibre samples to be tested were prepared in Japan, however a sub-set of samples were prepared at the Centre of Human and Aerospace Physiological Sciences at King's College London. The sets of fibres prepared in London were transported to the Spring-8 facility in Japan on dry ice, in a relaxing solution containing 75% glycerol. Upon arrival in Japan the samples were transferred to a relaxing solution containing 50% glycerol and stored at -20°C until the day of an experiment. Diffraction patterns obtained from sets of fibres which had been prepared in London were similar to those from sets of fibres which had been prepared in Japan, indicating that the transportation procedure did not affect the subsequent results.

On the day of an experiment, sets of fibres were washed in pre-activating solution (Figure 4.2) to remove the glycerol which the fibres had been stored in and placed into the specimen chamber. The sarcomere length of the fibres in a given set was adjusted to an optimum length of 2.75μm by changing the length between the two halves of the gold meshes, which subsequently altered the length of the fibres in the specimen chamber. The sarcomere length was measured using He-Ne laser diffraction, by shining the laser through a given set of fibres which was encased in the specimen chamber, and monitoring the diffraction spacing (1103P-1367, Uniphase, Manteca, Ca, USA, power = 4mW).

The specimen chamber was placed into the x-ray set up (Figure 5.2C) and immersed in pre-activating solution (Figure 4.2) with the addition of 5mM dithiothreitol, 200U of Creatine Phosphkinase (Sigma C755-35KU) and 1000U of catalase (Sigma C40). These enzymes served to scavenge hydrogen peroxide generated by the radiation during the x-ray diffraction experiment. A given set of fibres being tested was immersed in activating solution using a remotely controlled pump, which pumped activating

solution, also containing the aforementioned enzymes, through the set until the pre-activating solution had been removed. The activating solution used was solution A (Table 3.2), as this had been used in previous experiments conducted at the Spring-8 facility (Li et al., 2015).

X-ray diffraction patterns were recorded at 15°C. For each set of fibres, the diffraction patterns from the first ten exposures to the X-ray beam were used for analysis. The wavelength used was 0.1nm and the distances of the sets of fibres to the diffraction pattern camera detector (C4742, Hamamatsu Photonics; 1024 x 1024 pixels) was 2267mm and an image intensifier (VP5445, Hamamatsu photonics, Hamamatsu, Japan) was also used. The exposure time of the sets of fibres to the x-ray beam was kept low (two seconds) for each diffraction image taken, in order to reduce radiation damage during the experiment. The specimen chamber was moved so that diffraction patterns could be obtained every 100µm along the length of the fibres mounted in each set (Figure 5.2D). Furthermore, an aluminium attenuator 0.8mm thick was placed upstream of the specimen chamber. The beam flux was between 2.7 $x10^{11}$ and 4 $x10^{11}$ photons s^{-1} after attenuation, and the beam size at the sample position was 0.2mm (vertical) and 0.3mm (horizontal).

The intensity of the myosin meridional first layer line (MM1) reflection was recorded to give an estimate of the order of the array of the myosin cross-bridges during contraction of skinned fibres from young compared to elderly cohorts. The diffraction patterns obtained were taken and analysed by Dr Hiroyuki Iwamoto using specialised software at the Spring-8 facility. The principle of the diffraction pattern analysis was to summate the reflection intensities of the recorded diffraction patterns, followed by subtracting background scattering in order to visualise the diffraction pattern for a given experimental group.

5.2.7 Statistics

The scewness and kurtosis values of the mean specific force data did not differ significantly from normality and the Shapiro-Wilk test of normality indicated that the null hypothesis that the data were normally distributed could be accepted. The mean specific force values obtained from skinned fibres from each group in both solution A and solution B were analysed using a one-way ANOVA. The ratios of mean specific force in solution B compared to solution A and the mean MHC I skinned fibre myosin content was also analysed using a one-way ANOVA. Data are presented as means ± SEM. Statistical analysis was not carried out on the x-ray diffraction data, since the MM1 reflection intensity was summed for each group, meaning that there was only one value for each group.

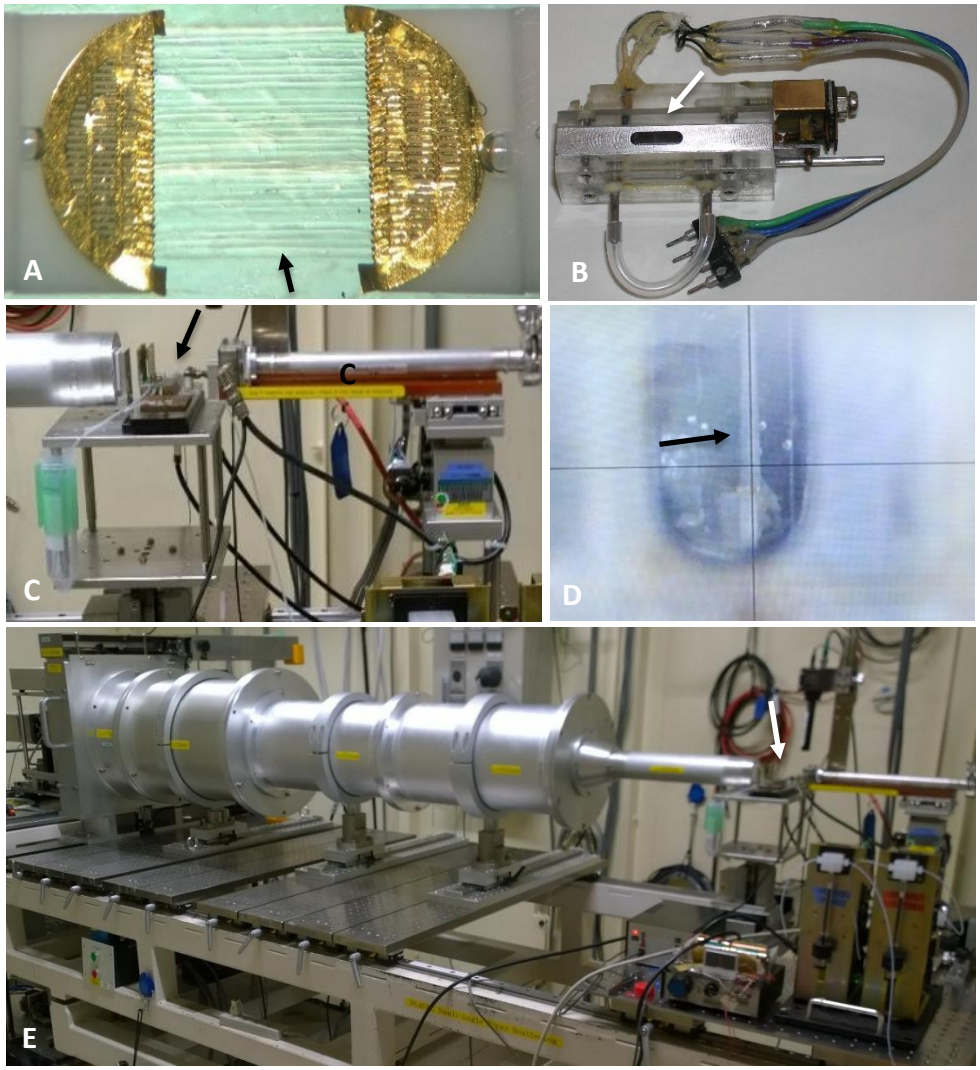


Figure 5.2. The different elements of the x-ray diffraction equipment at the BL45XU beamline, at the Spring-8 Synchrotron facility, Japan.

A: A set containing approximately thirty skinned fibres. Individual skinned fibres were clamped between two halves of a 3mm wide gold mesh which was attached to a ceramic chip. Approximately thirty fibres were mounted to each set.

B: The specimen chamber. A set of fibres was placed into the specimen chamber, pictured, positioned parallel to the opening (indicated by the white arrow), through which a laser could be shone to measure sarcomere length. The sarcomere length was adjusted by altering the length of the specimen chamber.

C: The position of the specimen chamber in the x-ray diffraction setup, indicated by the black arrow.

D: A side view of the specimen chamber viewed using a video camera. The skinned fibres are in a vertical position and are indicated by the black arrow. The centre of the black cross in the image indicates where the x-ray beam will be focused through the skinned fibres.

E: the column through which the scattered x-ray beam travels before the diffraction pattern is recorded.

5.3 Results

5.3.1 Fibre type distribution

The number and proportion of fibres studied of each type in each experimental group is illustrated in Figure 5.3. A total of ninety-eight fibres were studied from the young group, and ninety-two and ninety-seven fibres were studied from the master cyclists and hip fracture patients respectively. There was a relatively even proportion of MHC I (42.8%) and MHC IIA (44.9%) fibres studied in the young group. In contrast, 85.9% of fibres studied from master cyclists and 72.2% of fibres studied from hip fracture patients were MHC I fibres. Due to the small number of MHC IIA and hybrid fibres, but relatively large number and proportion of MHC I fibres in the master cyclist and hip fracture patient groups (Figure 5.3), statistical comparisons of skinned fibre specific force between groups have only been made using MHC I fibres.

5.3.2 Variability of specific force measurements within individuals

Considerable variability was observed in specific force measurements taken from MHC I fibres within the individual participants studied. The average range of skinned fibre specific force values was calculated by dividing the maximum specific force recorded for a given individual, by the minimum specific force, which yielded the range of specific force values as a fold-difference. In solution A, within individuals, specific force varied 5.9 ± 6.5-fold for the young group, 5.1 ± 2.2-fold for master cyclists and 6.5 ± 5.4-fold for hip fracture patients. In solution B the average fold differences were 4.6 ± 4.3 in the young group, 6.3 ± 4 for master cyclists and 7.5 ± 5.9 for hip fracture patients. Therefore, the variability in specific force values was slightly lower when fibres contracted in solution B, compared to solution A in the young group, but slightly higher

in solution B compared to solution A in fibres from master cyclists and hip fracture patients. Nevertheless, considerable variability in skinned fibre specific force measurements was observed in skinned fibres from individuals of all experimental groups.

5.3.3 The trends in mean specific force data between groups

The mean MHC I skinned fibre specific force measured in solution B was significantly greater than that measured in solution A, for all groups studied. However, between groups, specific force measured in solution A was no different between young participants, master cyclists or hip fracture patients (Figure 5.5) ($p > 0.05$) and there was no discernible trend for specific force to be higher or lower in a given group. In contrast, there was a trend for specific force measured from the same MHC I fibres in solution B to be highest in young participants, followed by master cyclists, and lowest in hip fracture patients, although no statistically significant differences were observed ($p > 0.05$).

Interestingly, the MHC I fibre specific force measured from the same fibres in solution A compared to solution B, exhibited a different degree of change in the different groups. Skinned fibre specific force from young individuals increased almost two-fold in solution B compared to solution A. The differences in specific force between solutions A and B were not as great in skinned fibres from the elderly cohorts, increasing 1.65-fold in master cyclists compared with just 1.38-fold in hip fracture patients (Figure 5.6). However, despite the trend observed, the differences in MHC I skinned fibre response between solutions A and B across the different experimental groups were not significantly different ($p > 0.05$).

	Young		**Master Cyclists**		**Hip Fracture Patients**		
Subject	*Gender*	*Age*	*Gender*	*Age*	*Gender*	*Age*	*History of major illness (other)*
1	Female	21	Female	79	Female	78	Breast cancer, lymphoma
2	Female	32	Female	76	Female	59	None
3	Male	22	Male	75	Female	77	COPD (multiple falls in the last year)
4	Male	25	Male	73	Female	82	Hypertension, rheumatoid arthritis, type 2 diabetes
5	Male	29	Male	71	Male	77	Rheumatoid arthritis
Mean age		**25.8**		**74.8**		**74.6**	

Table 5.1. Gender and age of male and female participants studied, with the addition of any history of major illness in the hip fracture patient cohort.

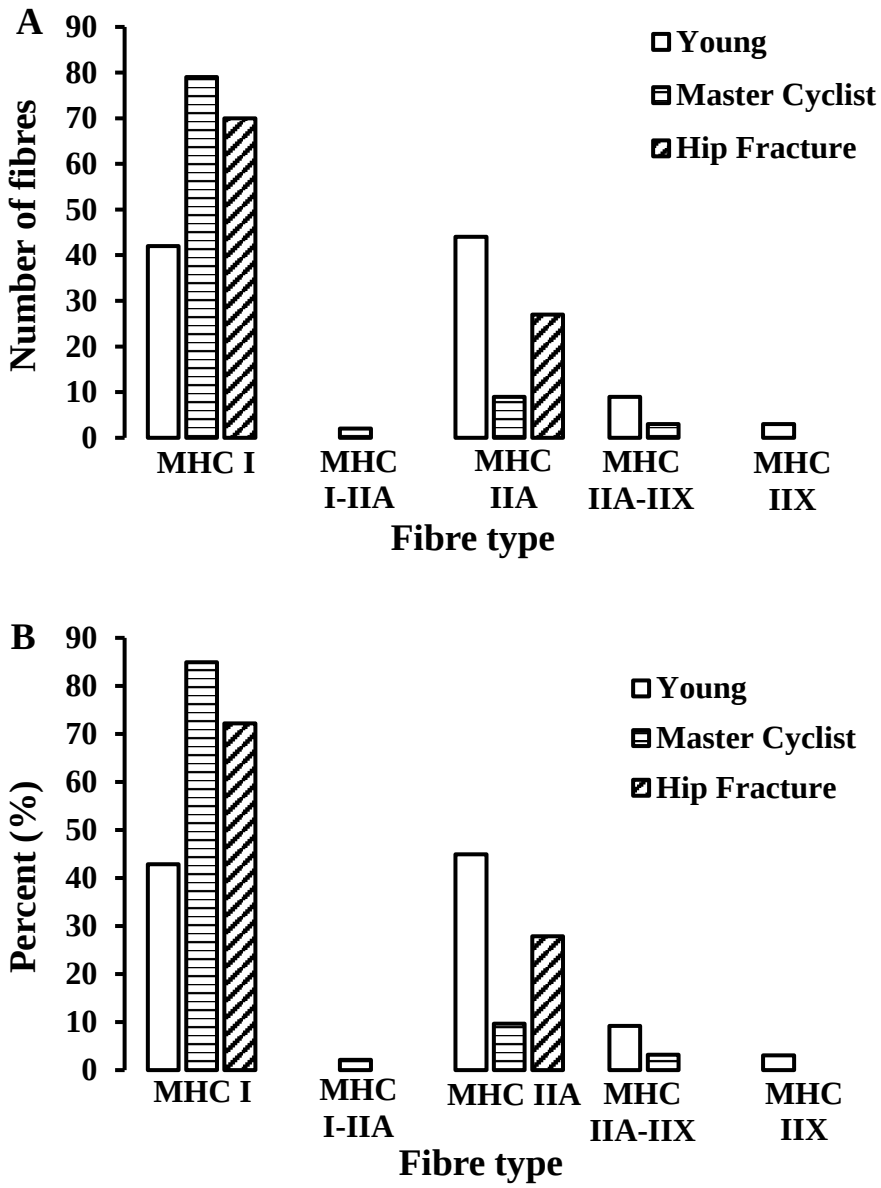


Figure 5.3. A: The number of fibres of a given MHC isoform within each experimental group.
There was a relatively even distribution of MHC I (n = 42) and MHC IIA (n = 44) fibres in young participants. In both master cyclists and hip fracture patients there was a predominance of MHC I fibres, being 79 of 92 and 70 of 97 fibres in each group respectively. **B:** The relative proportion of the total number of each fibre type in each group. There was a relatively even proportion between MHC I (42.8%) and MHC IIA (44.9%) fibres from young participants, whereas master cyclists and hip fracture patients exhibited predominant 85.9% and 72.2% proportions of type I fibres respectively.

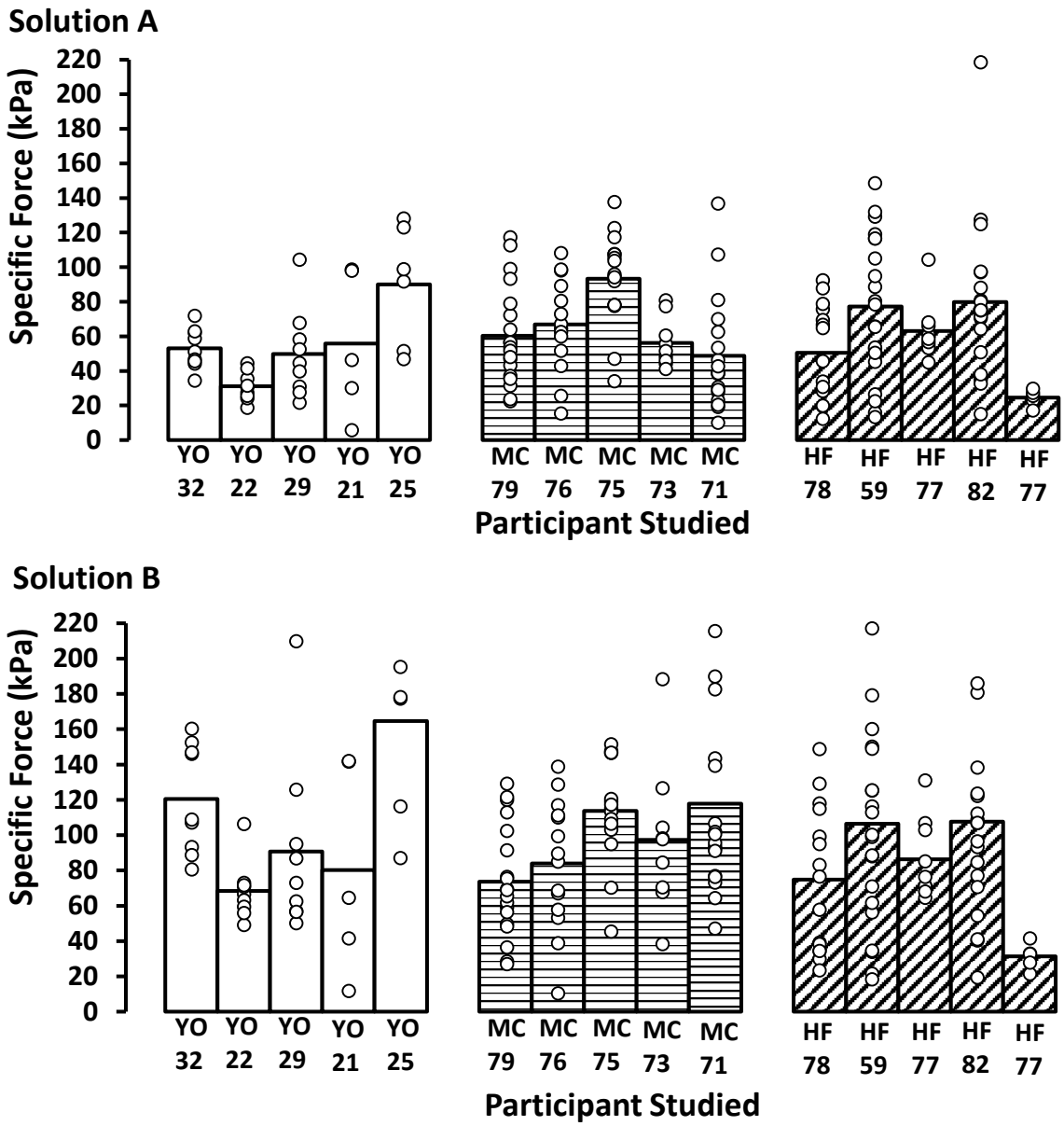


Figure 5.4. Specific force of MHC I fibres of individual participants from young (YO), master cyclist (MC) and hip fracture patient (HF) experimental groups in solution A and solution B.

The variation in MHC I fibre specific force for each individual subject is displayed by the individual data points which correspond to specific force data from a single fibre. The average fold-difference in specific force for each group in solution A was, YO: 5.9 ± 6.5, MC: 5.1 ± 2.2, and HF: 6.5 ± 5.4 fold-differences. In solution B, the variation fold-differences for each group was: YO: 4.6 ± 4.3, MC: 6.3 ± 4, and HF: 7.5 ± 5.9. Data are shown as mean values (bars) as well as individual data points (white circles).

5.3.4 Skinned fibre myosin content

That the BAP of MHC bands corresponding to a given skinned fibre sample tested using SDS PAGE, is proportional to the myosin content in the sample, was demonstrated in section 3.12. Whether the Laemmli Sample Buffer (LSB) had a homogeneous effect across the whole of a given skinned fibre sample and between different samples, with regard to myosin extraction, needed to be verified. Furthermore, the use of two batches of LSB meant that the effect of both batches on skinned fibre samples needed to be tested.

Two different volumes of the same skinned fibre samples exhibited the same BAP per µl of sample loaded onto a gel, indicating that the effect of the first batch of LSB was homogeneous throughout a given skinned fibre sample (Figure 5.7). Comparing the two different batches of LSB, both batches demonstrated a consistent effect on the myosin standard sample, since the myosin standard curves obtained using each batch of LSB on the same gel were virtually identical (Figure 5.8A). Furthermore, myosin content per unit of fibre volume, quantified from two different halves of the same skinned fibre, both of which had been processed in either the new or old batch of LSB, were similar. This indicates that the effect of each batch of LSB was homogeneous across different samples of skinned fibres (Figure 5.8B).

The absolute myosin content of the skinned fibres examined was normalised to fibre volume, to provide a measurement of myosin content per unit of fibre volume. Consistent with the previously mentioned specific force values, variability was also observed in MHC I skinned fibre myosin content within individuals from each experimental group. The mean range of variation in MHC I fibre myosin content was 3.25 ± 0.6-fold in young participants, 9.4 ± 4.8-fold in master cyclists and 5.8 ± 2.3-fold in hip fracture patients (Figure 5.9). Overall, the mean values for MHC I fibre myosin content

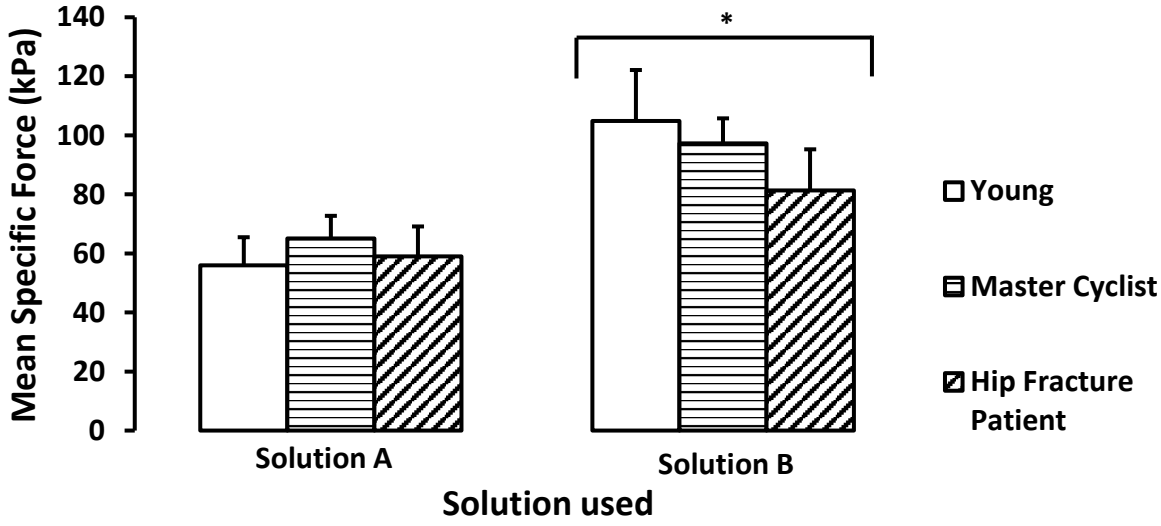


Figure 5.5. Mean specific force of each experimental group, measured from the same fibres tested in both solution A and solution B.
Young: *n* = 85; Master Cyclists: *n* = 85; Hip fracture patients: *n* = 99. Specific force was significantly higher in the same MHC I fibres studied in solution B compared to solution A in all groups, as indicated by the asterisk. A difference in trend was observed from the same fibres activated in each experimental solution. In solution A, specific force was similar between experimental groups, whereas in solution B, specific force was highest in young individuals and lowest in hip fracture patients. However, no statistically significant differences were observed in mean specific force between groups in either solution A or B. Data are means ± SEM.

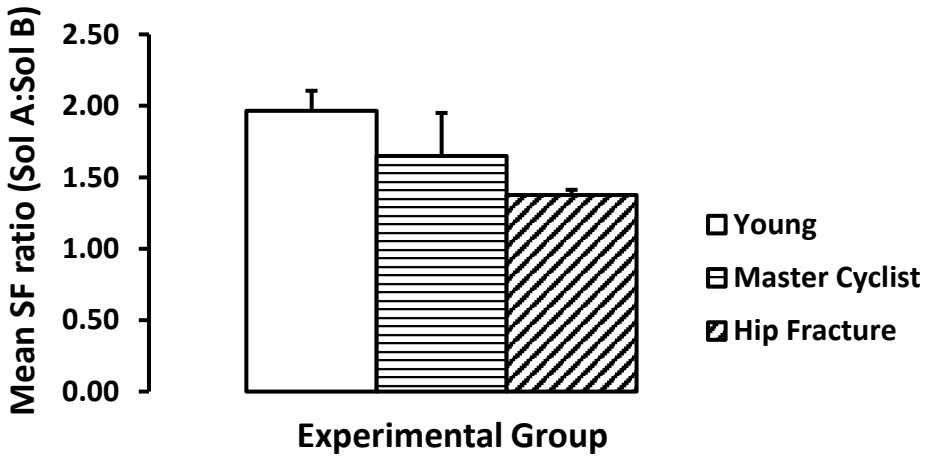


Figure 5.6. The mean ratio of specific force in solution B:specific force in solution A, for each experimental group.
Young: *n* = 85; Master Cyclists: *n* = 85; Hip fracture patients: *n* = 99. The value of the ratio indicates that in young individuals specific force was approximately double (1.97 times greater) in solution B compared with the same fibres activated in solution A. The specific force increase from the fibres contracting in solution B, compared with the same fibres in solution A, was not as great in elderly individuals, being 1.65 and 1.38 times greater in master cyclists and hip fracture patients respectively. Data are means ± SEM.

were similar between all three experimental groups, with no statistically significant differences observed (Figure 5.10).

The mean MHC I skinned fibre myosin content of each experimental group was plotted against the corresponding mean specific force measured from the same fibres, in order to assess the relationship between these two variables in each group. Due to the similar MHC I fibre myosin content and specific force across all groups studied, a clustering of mean data points was observed (Figure 5.11). The relation between myosin content and specific force from individual fibres is illustrated in section 8.3.

Correlations were made between myosin content and specific force from the same skinned fibres. The mean correlations for each experimental group revealed that there was no relationship between myosin content and specific force measured in solution A (R^2 = 0.08) or solution B (R^2 = 0.09) in fibres from master cyclists. The mean correlation between type I fibre specific force and myosin content demonstrated a weak relationship in both solution A (R^2 = 0.16) and solution B (R^2 = 0.18) in the hip fracture patient group. In contrast to the two elderly groups, the mean correlation between myosin content and MHC I fibre specific force was relatively high in the young group for specific force measurements taken both in solution A (R^2 = 0.50) and solution B (R^2 = 0.46) (Figure 5.12).

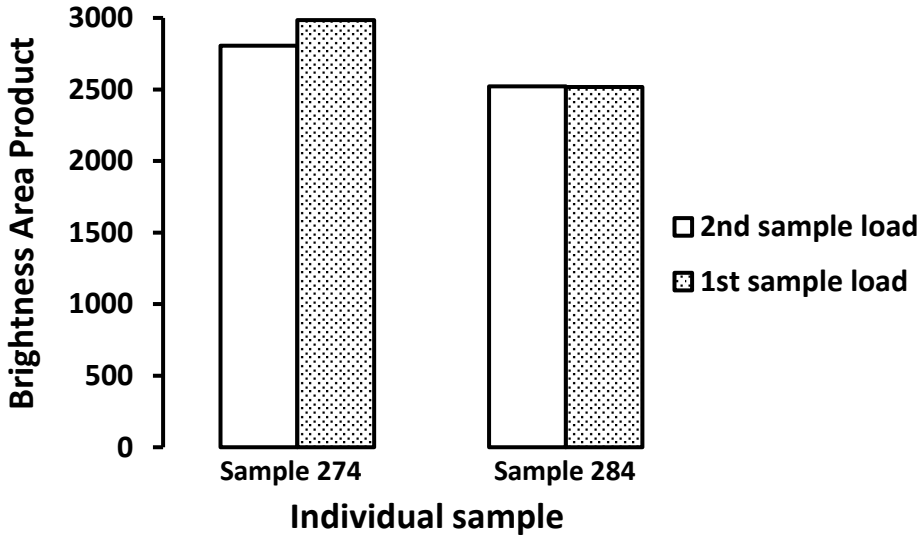


Figure 5.7. The BAP per µl of sample loaded onto an SDS PAGE gel, plotted for two different sample loads of the same skinned fibre.
Two skinned fibres were used to assess this relationship. The BAP per µl of sample was in close agreement for both sample loads for each skinned fibre tested, within 6% for sample 274 and 1% for sample 284.

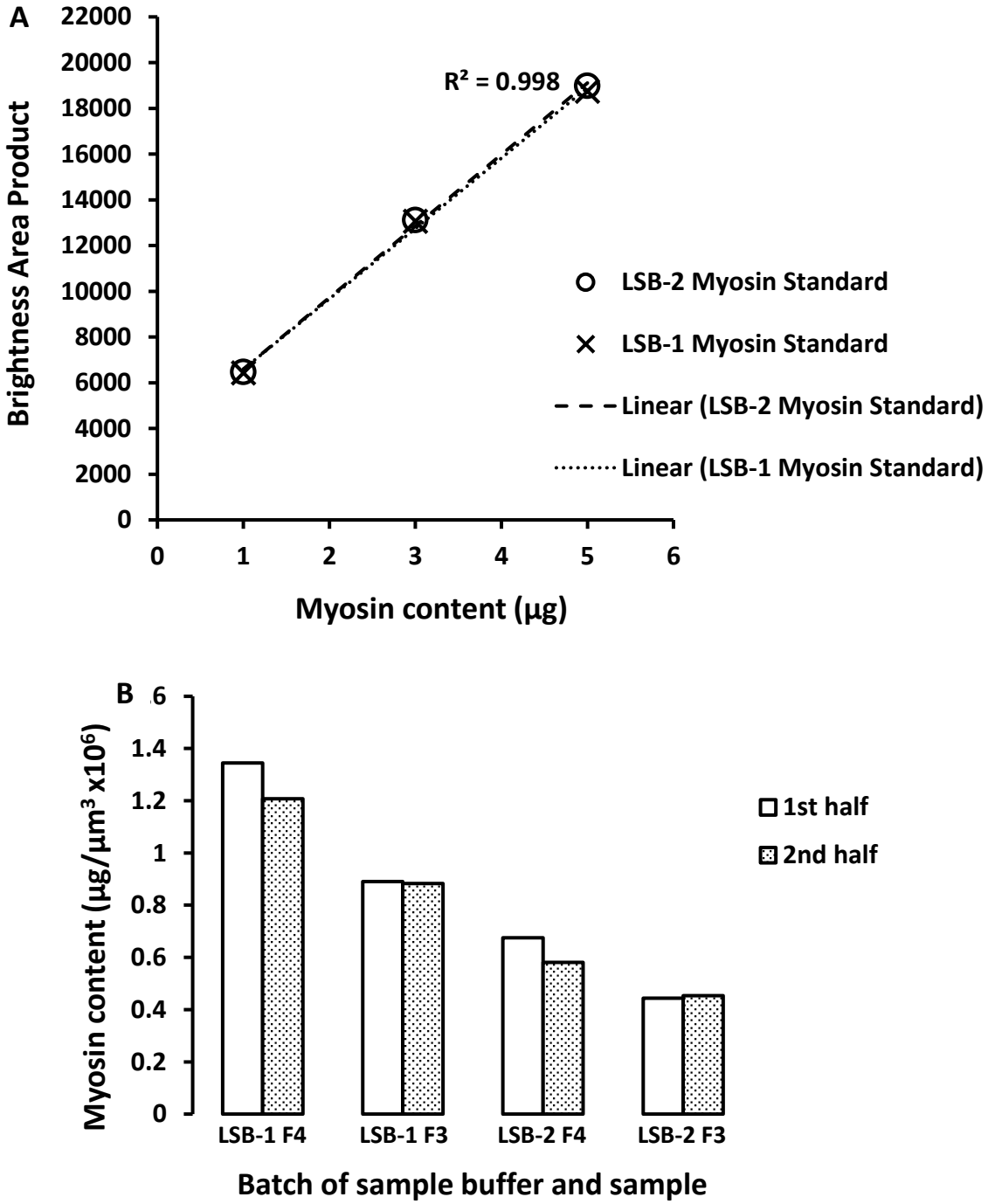


Figure 5.8. Testing the effect of two different batches of Laemmli sample buffer (LSB) on the myosin standard sample (A), and two halves of the same skinned fibres (B).

A: The myosin standard curve plotted when the myosin standard sample was processed in either the first (LSB-1) or second (LSB-2) batch of LSB. The two curves are almost identical and both exhibit a highly linear relationship between myosin content and the corresponding BAP within the range of 1µg-5µg of myosin.

B: The myosin content in each half of the same skinned fibre, processed in either the first or second batch of LSB. There was high agreement in the myosin content values of two halves of the same fibre, within each batch.

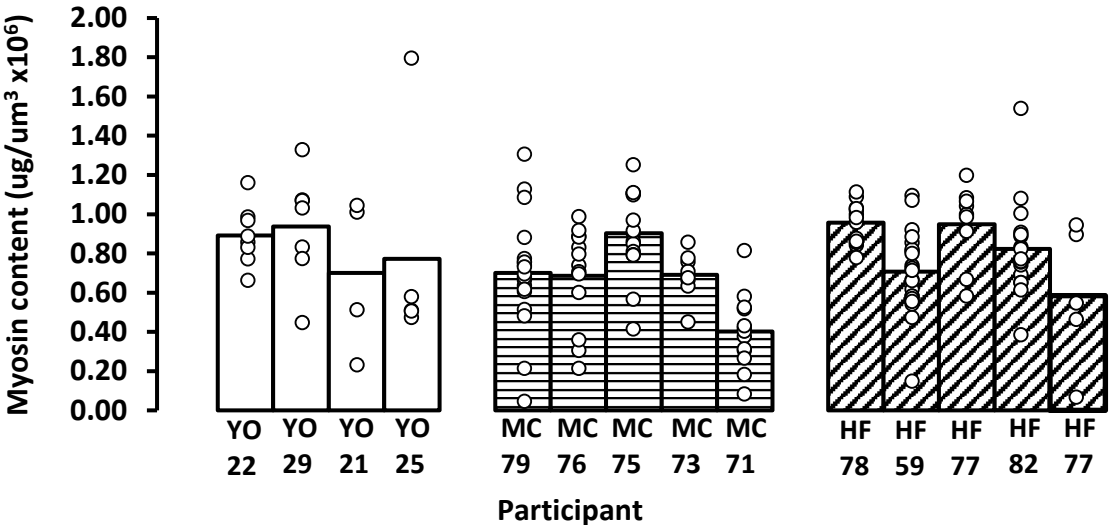


Figure 5.9. Mean type I fibre myosin content of individual participants from each experimental group.

Young: *n* = 24; Master cyclists: *n* = 68; Hip Fracture patients: *n* = 65. The variation in type I fibre myosin content for each individual subject is displayed by the individual data points which correspond to specific force data from a single fibre. The average variation (mean ± SEM) was 3.25 ± 0.6-fold for the young group, 9.4 ± 4.8 and 5.8 ± 2.3-fold for master cyclists and hip fracture patients respectively.

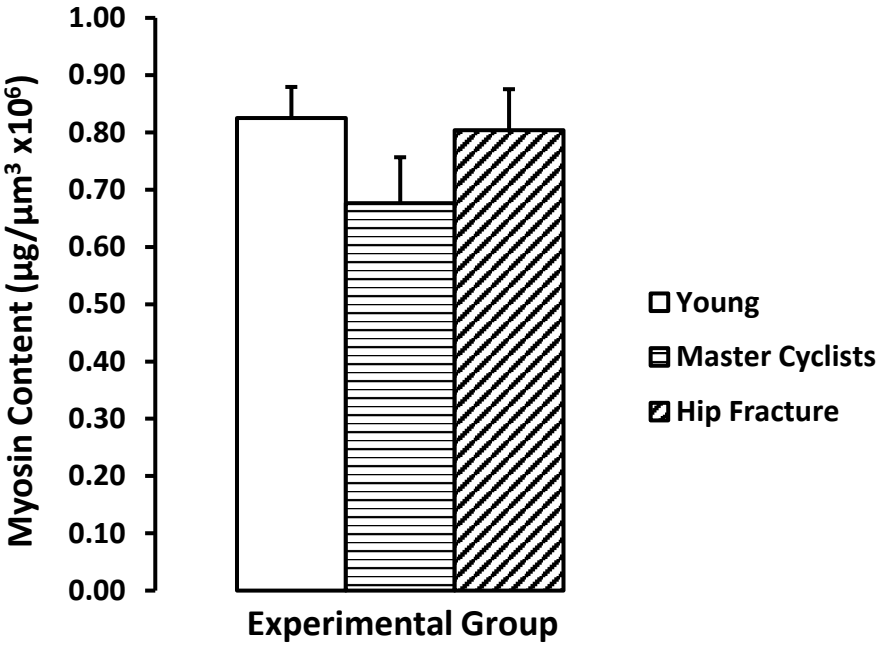


Figure 5.10. Mean skinned fibre myosin content of each experimental group. Young: *n* = 24; Master cyclists: *n* = 68; Hip Fracture patients: *n* = 65. No statistically significant difference in myosin content was observed. Data are means ± SEM.

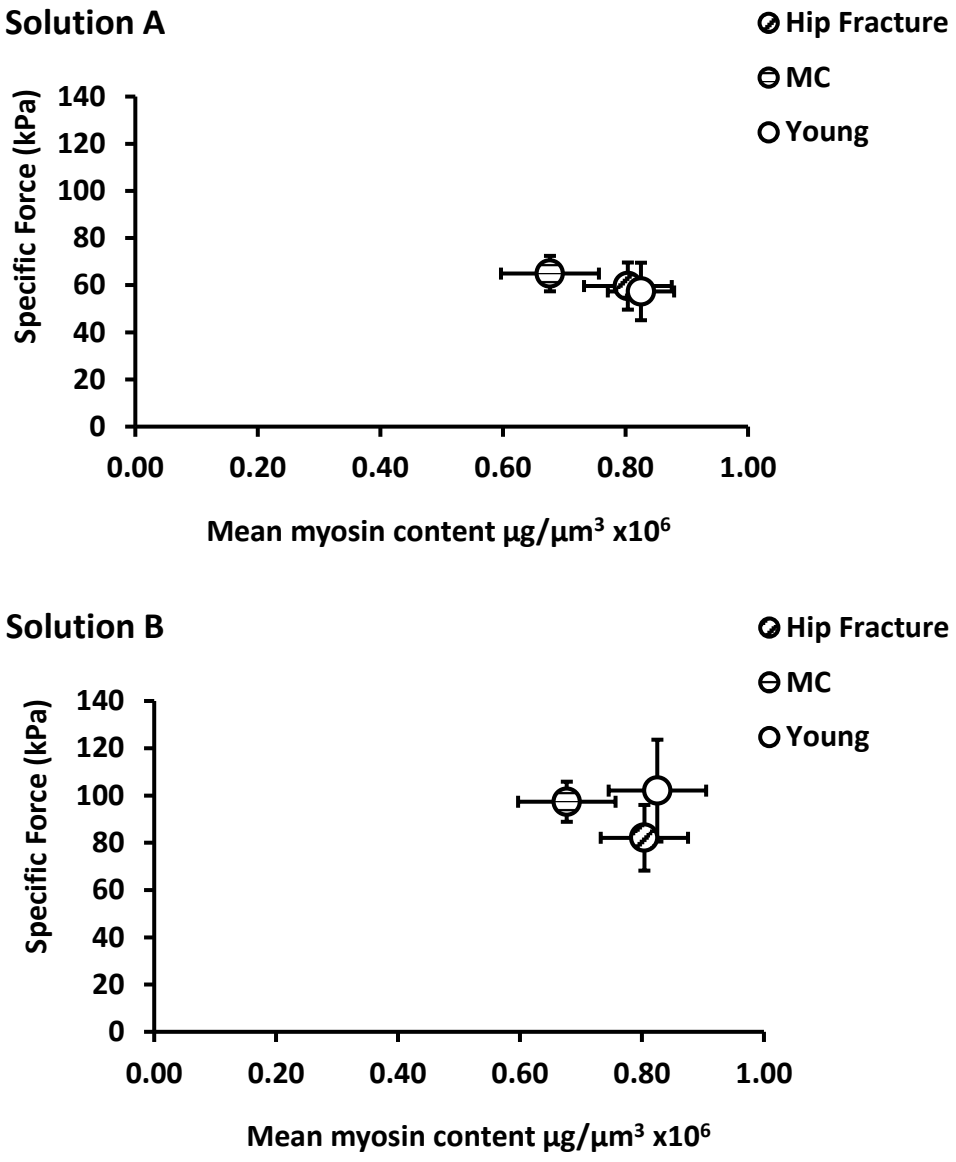


Figure 5.11. Mean MHC I fibre specific force vs mean MHC I fibre myosin content for each experimental group.

The similar mean myosin and specific force values for each group cause the data points to be grouped together, indicating that the relationship between myosin and specific force was similar between young, master cyclist and hip fracture patient groups. The vertical error bars represent the SEM of specific force and the horizontal error bars represent the horizontal SEM of myosin content.

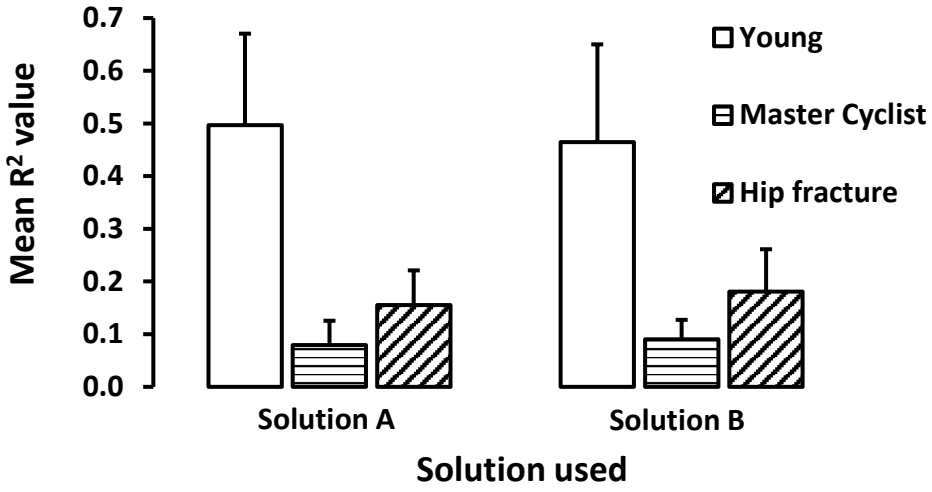


Figure 5.12. The mean of the correlation between specific force and myosin content within individuals of a given experimental group.
The relationship was strongest in the young group in both solution A (R^2 = 0.50) and solution B (R^2 = 0.46), whereas a weak relationship was observed in hip fracture patients in both solution A (R^2 = 0.16) or solution B (R^2 = 0.18) and no relationship was observed in master cyclists in either solution A (R^2 = 0.08) or solution B (R^2 = 0.09). Data are means ± SEM.

5.3.5 X-ray diffraction: myosin meridional first order layer line

Numerous sets of ~30 fibres were mounted for each experimental group. The X-ray diffraction recordings from all sets used from each group were summed together. The mean value of the MM1 reflection intensity was quantified for each experimental group studied, yielding one value for all sets of fibres tested from each group in pre-activating solution compared to activating solution. The MM1 reflection intensities are expressed as the ratio of MM1 to the sixth actin layer line. Due to there being one value per group, statistical analysis was not conducted on the preliminary data obtained. The ratio of the reflection intensities for the young group in pre-activating and activating solution were 0.0679 and 0.096 respectively. The ratio of the reflection intensities for the master cyclists in pre-activating and activating solution were 0.1076 and 0.1867 respectively. The reflection intensity ratios for the hip fracture patients in pre-activating and activating solution were 0.0726 and 0.1463 respectively.

The intensity profile of the MM1 reflection was increased during skinned fibre contraction compared to when the fibres were in a relaxed state in all groups studied. Based on the physical principles of X-ray diffraction, a higher reflection intensity is caused by a higher order of the molecules in a given sample. In the present study, the samples were sets of approximately thirty skinned muscle fibres from either young individuals, master cyclists or one hip fracture patient. Since the MM1 reflection corresponds to the 14.3nm axial repeat of the myosin filament head (Huxley and Brown, 1967), an increased MM1 reflection suggests that a greater array of myosin heads are aligned in an ordered fashion during contraction, compared to a relaxed state. The MM1 reflection intensity was similar between groups during contraction, indicating that the order of myosin filament heads was similar between the young and elderly cohorts.

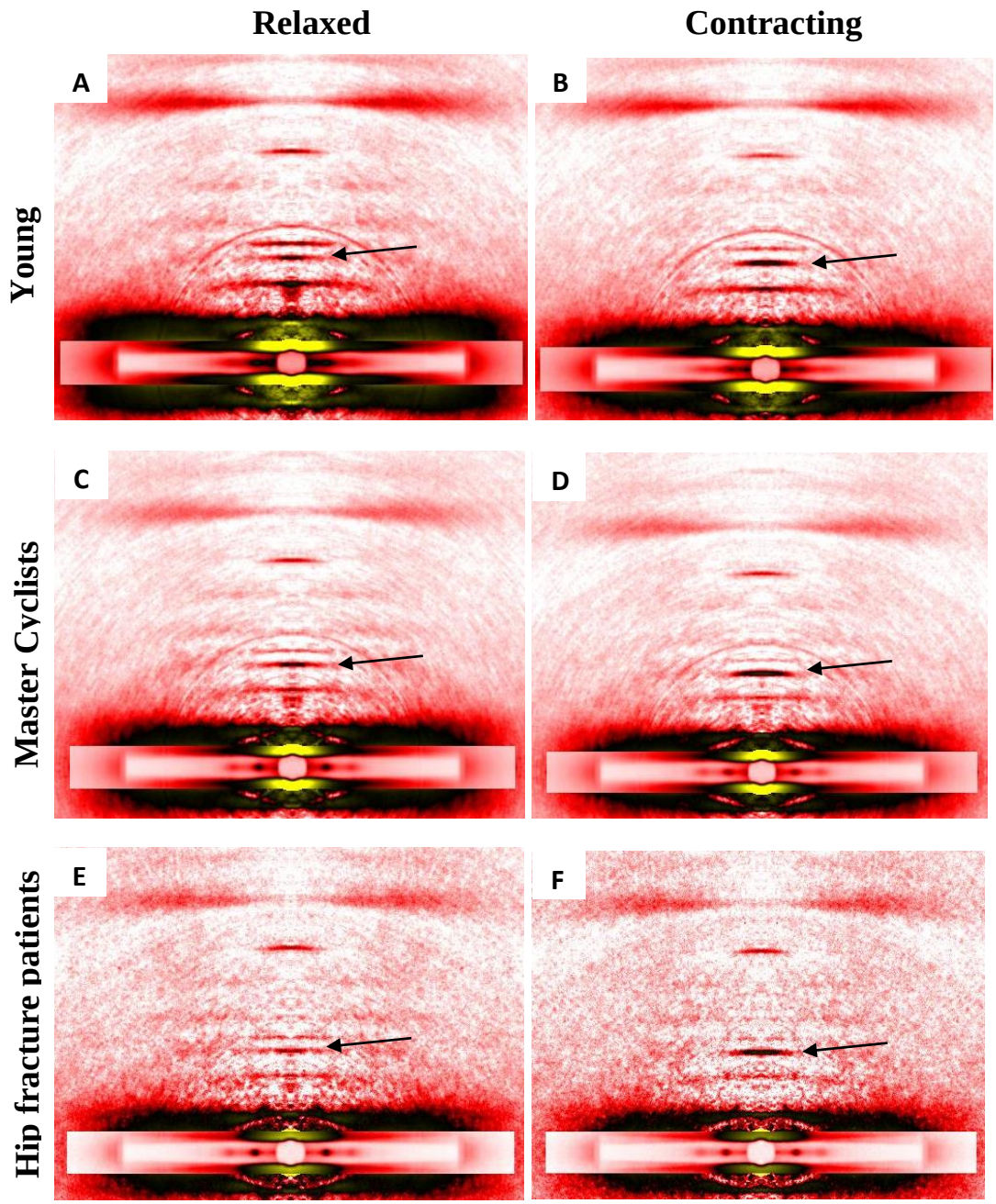


Figure 5.13. Low angle x-ray diffraction reflections from young participants (A & B), master cyclists (C & D), and one hip fracture patient (patient 1) (E & F).
The age and gender of all participants are in
Table **5.1**. The sets of fibres in a relaxed state in pre-activating solution are in panels A, C and E. The diffraction patterns from the sets of fibres during isometric contractions in activating solution A are in panels B, D and F. The reflection intensity profile of the MM1 reflection, pointed out by the black arrow in all panels, was increased during skinned fibre contraction in activating solution in all groups compared to when fibres were relaxed in pre-activating solution. The diffraction patterns from hip fracture patient skinned fibres (panels E & F) contain a greater amount of background noise due to the low number of samples available.

5.4 Discussion

The main findings of the present study showed that neither age nor physical activity appear to affect skinned fibre specific force, but that specific force varied between individuals and was observably different between solutions. However, the skinned fibres tested exhibited markedly different trends between the cohorts studied, depending on the activating solutions used to study skinned fibres, despite statistically significant differences not being observed. This implies that the conclusion drawn from a given study regarding skinned fibre specific force could be dependent on the activating solution used. Furthermore, the present study's findings support that skinned fibre specific force across different populations is modulated by myofibrillar protein content.

The comparison of specific force and myosin content measurements between the three experimental groups studied in the present investigation was made using MHC I fibres, due to the low number of MHC IIA fibres in the elderly groups (Figure 5.3). Skinned fibre specific force from MHC I fibres was found to be variable within individual subjects in all groups studied (Figure 5.4). Despite the significant difference in specific force observed from the same fibres contracting in solution A compared to solution B, the variability in the specific force values did not differ substantially between the two solutions, as indicated by the range of values obtained. Specific force data were included based on the strict criteria of consistency of skinned fibre force production over five consecutive contractions, as opposed to a criteria which arbitrarily excluded force values. This may have increased the range of specific force values analysed, although three-fold variability in MHC I fibre specific force within the same study has also been observed elsewhere (Gilliver et al., 2009).

5.4.1 The trends in mean specific force results measured in solutions A and B

5.4.1.1 Solution A

In the present study, mean MHC I skinned fibre specific force was similar between young, master cyclist and hip fracture patient groups, when fibres were tested in solution A. The observation that there was no significant difference between specific force from master cyclists and young individuals is consistent with previous findings that exercise maintains skinned fibre specific force in the elderly (D'Antona et al., 2007, Korhonen et al., 2006). However, specific force was not significantly lower in comparatively frail hip fracture patients compared to young participants. This finding contradicts the hypothesis that physical activity prevents skinned fibre specific force loss in the elderly and is consistent with previous work which reported that skinned fibre specific force was similar between mobility-limited (Reid et al., 2012) and wheelchair bound (Venturelli et al., 2015) elderly cohorts.

The physical activity profile of the hip fracture patients in the present study was not documented, but was assumed to be lower than the master cyclists' physical activity profile, which had been carefully characterised as part of previous work (Pollock et al., 2015). Therefore, if physical activity ameliorates specific force loss, the lack of a difference between master cyclists and hip fracture patients could potentially be due to the latter being a physically active cohort prior to requiring surgery, although this is unlikely given their history of major illness (Table 5.1). Overall, the lack of difference between the physically active and comparatively unhealthy elderly groups with the young cohort, suggests that neither ageing nor daily physical activity in the elderly affects specific force at the level of myofilament interaction, at least up to the age range studied.

An interesting point to consider is the amount of physical activity required to ameliorate the onset of specific force loss at the skinned fibre level. Several investigations have reported similar specific force values in physically inactive elderly compared to young cohorts, such as mobility-limited (Reid et al., 2012) or chronically wheel chair bound (Venturelli et al., 2015) individuals. In contrast, young and elderly individuals who have undergone an extreme duration of non-weight bearing have consistently exhibited specific force loss at the skinned fibre level (D'Antona et al., 2003, Trappe et al., 2004, Trappe et al., 2008, Larsson et al., 1996). This suggests that an extreme intervention such as the complete absence of weight bearing is required for a prolonged period of time to reduce the intrinsic force generating capacity of individual skeletal muscle fibres, as opposed to simply inducing functional changes associated with reductions in fibre CSA. Indeed, resistance training of long duration (Pansarasa et al., 2009, Parente et al., 2008**),** but not short duration (Trappe et al., 2000) has caused improvements in skinned fibre specific force, supporting that a sufficiently strong stimulus is required for long periods of time to induce changes to a muscle fibre's intrinsic force generating potential. Therefore, perhaps even chronic inactivity in elderly individuals is not sufficient to mimic the effects of a complete absence of weight bearing, which is itself used to be a model of accelerated ageing.

5.4.1.2 Solution B

In contrast to the lack of a clear trend in specific force between groups in solution A, a trend was observed, albeit not statistically significant, for specific force to be highest in young individuals, followed by master cyclists, and lowest in the hip fracture patients when the same MHC I skinned fibres contracted in solution B. The trend suggests that specific force is relatively preserved in endurance trained compared to frail elderly individuals, supporting previous findings that physical activity modulates skinned fibre

specific force in the elderly (D'Antona et al., 2007). The lack of statistical significance is likely to be related to the variation in skinned fibre specific force observed within individuals. Another reason could be that the present study was underpowered. Indeed, almost double the number of MHC I fibres were tested in elderly compared to young individuals, due to the predominance of MHC I fibres in the elderly groups and relatively even proportion of MHC I and IIA fibres in the young group. Therefore, a higher number of MHC I fibres from young individuals may affect the statistical significance of the observed trend.

Although not statistically significant, the clear difference in trend observed in specific force measured from the same fibres in solutions A and B suggests that different conclusions might be drawn from the same study depending on the activating solution used. This implies that different experimental solutions used in different studies are likely to have contributed to the equivocal results regarding the occurrence of specific force loss in sedentary elderly compared to young cohorts (Larsson et al., 1997, Yu et al., 2007, Ochala et al., 2007, Raue et al., 2009, Trappe et al., 2003, Hvid et al., 2011, Frontera et al., 2003, Frontera et al., 2000a) as well as the equivocal results regarding whether physical activity or inactivity in the elderly modulates specific force (D'Antona et al., 2003, D'Antona et al., 2007, Power et al., 2016, Reid et al., 2012, Venturelli et al., 2015).

Information detailing the chemical constituents of the activating solutions used by studies measuring specific force from human skinned fibres could be compiled from the studies included in the systematic review described in Chapter 2. 75% of data sets compiled in Chapter 2 used Imidazole as a pH buffer. In Chapter 4, Imidazole was shown to negatively affect skinned fibre specific force. The fact that the majority of the literature uses Imidazole suggests that this particular compound does not cause the equivocal results regarding the occurrence of age-related specific force loss. However, clearly the majority

of publications on human skinned fibres have studied fibres contracting sub-optimally, by using Imidazole as a pH buffer.

The hypothesis that the experimental conditions during contractile measurements from skinned fibres can influence the conclusions drawn regarding specific force from a given data set, is demonstrated by the two different observations made in the same study by Callahan et al. (2014). MHC I and IIA Vastus Lateralis fibre specific force measured at 25°C was not significantly different between elderly osteoarthritic and healthy elderly controls. However, MHC IIA specific force was significantly lower in the osteoarthritic group when measured at 15°C, albeit from a separate set of fibres. Therefore, experimental conditions other than the activating solution used, such as temperature, might well affect the conclusions drawn from skinned fibre specific force measurements.

In addition to experimental conditions affecting the conclusions drawn from a given study, it has been suggested that using CSA as a proxy to represent the sarcomeres in parallel in a single muscle fibre is inappropriate. Toth et al. (2012) observed no change in MHC I and IIA Vastus Lateralis specific force measured at 15°C and 25°C, following eighteen weeks of resistance training in elderly men and women, when skinned fibre P_0 was normalised to CSA. In contrast, normalising P_0 to the myofibrillar fraction, calculated from EM images, revealed that specific force measured at 25°C was significantly increased in MHC I and IIA fibres following resistance training. However, the myofibrillar fraction was extrapolated using the EM data from a sub-set of the participants studied, so must be interpreted with caution. Even so, skinned fibre CSA measurements are also confounded by the variable effects of swelling which occurs due to chemical skinning (discussed in section 2.1.6), indicating that CSA measurements and subsequently, representations of sarcomeres in parallel, may be inaccurate.

5.4.2 The implications of the differences in force measured between solutions A and B

The difference in force between solutions A and B measured in the same skinned fibres indicates that despite reaching a plateau in force production during an isometric contraction, specific force measured in solution A is clearly not maximal. This could have implications for other measurements taken relating to cross-bridge behaviour, such as stiffness measurements. However, force may still be proportional to stiffness in solution A, even if neither force nor stiffness measurements are maximal. An investigation into the relationship between skinned fibre stiffness and force in solutions A and B, given the relationship between the two variables in human skinned fibres (Ochala and Larsson, 2008) could provide critical information for understanding the cause of the increase in specific force observed in solution B.

A novel finding of the present study is that the trend in specific force from the same skinned fibres between young and elderly cohorts differs depending on the activating solution used. However, this trend was not statistically significant, which may be due to the variability in specific force measurements from each individual. Interestingly, the difference in skinned fibre specific force between solutions A and B had a tendency to be greater in young compared to elderly individuals. The fact that the specific force from the same fibres tends to increase more in fibres from young individuals contracting in solution B, compared to the hip fracture patients in particular, could suggest that skeletal muscle fibres from different cohorts exhibit differences in cross-bridge behaviour across the two activating solutions used (Figure 5.6). Indeed, specific force was approximately double (1.97 times) in solution B compared to in solution A in skinned fibres from young participants. In contrast, the increase in specific force from solution A to B was lower in master cyclists (1.65 times) and even lower in hip fracture patients (1.38 times), although

this trend was not statistically significant. The lack of statistical significance may be related to statistical power. Indeed, when either the individual data points from each group were compared using a one-way ANOVA, or an independent t-test was used to compare young individuals and hip fracture patients only, statistical significance was observed. Therefore, perhaps having five individuals per group, and subsequently five mean values to compare in a one-way ANOVA, means that the analysis is not sufficiently powered, causing the lack of significance. The potential mechanism underpinning the observed trends between specific force measured in solutions A and B is discussed below, in light of the MHC I skinned fibre myosin content and MM1 reflections obtained from low angle x-ray diffraction.

5.4.3 Skinned fibre myosin content

The linearity of the myosin standard curve (Figure 3.4), and the homogeneous effect of both batches of LSB within samples (Figure 5.7) and across different samples (Figure 5.8) indicates that any relative differences in myosin content between groups could be reliably detected. The mean MHC I skinned fibre myosin content was similar between young participants, master cyclists and hip fracture patients (Figure 5.10), which is consistent with the similar MHC I fibre specific force between the groups in solutions A and B (Figure 5.5). Therefore, the findings from the present study support previously published observations from skinned fibres (D'Antona et al., 2003) and homogenate muscle samples (Trappe et al., 2003) that differences in skinned fibre specific force between groups are dependent on differences in myofibrillar protein content. This is illustrated in Figure 5.11, where a clustering of data points is observed due to the similarities between specific force and myosin content between young and elderly cohorts. If specific force loss had been observed in the present study, an associated

reduction in skinned fibre myosin content, similar to that published by D'Antona et al. (2003) (see Figure 1.8) , would be expected.

Interestingly, within individual subjects from a given experimental group the mean correlation between specific force and myosin content was considerably stronger in young participants, compared to master cyclists or hip fracture patients (Figure 5.12). However, the myosin content quantified for a given individual fibre does not differentiate between different parts of the myosin molecule, so this correlation is not necessarily indicative of the relationship between the behaviour of the available myosin heads forming cross-bridges, and specific force. Furthermore, measurements reflecting skinned fibre cross-bridge stiffness and behaviour were beyond the scope of the present study, meaning that direct assessments of how myosin may be behaving differently in young compared to elderly participants cannot be made. Therefore, the significance of the higher correlation between specific force and myosin content in skinned fibres is difficult to ascertain without further experiments.

A technical point to make about the myosin content data, is that the accuracy of the myosin content normalised to skinned fibre volume could be improved by more accurate volume measurements. As described in section 3.8, skinned fibre volume was calculated from fibre length measured at x10 magnification using an eyepiece graticule, and depth and diameter measurements measured at x40 magnification. Geiger et al. (2000) also used light microscopy to measure skinned fibre volume, but corrected their measurements using a correction factor, calculated from the degree of error between their light microscope depth measurements compared to a confocal microscope. A separate experiment would need to be conducted to obtain the appropriate correction factor, but would be useful in confirming the accuracy of the trends in skinned fibre myosin content observed between groups in the present study.

5.4.3.1 The potential contribution of a reduced number of cross-bridges, or a difference in cross-bridge behaviour, to the observed trends.

Specific force has been shown to be proportional to myofibrillar protein content as well as skinned fibre stiffness (D'Antona et al., 2003, Ochala and Larsson, 2008), implying that the number of available acto-myosin cross-bridges is dependent on myofibrillar protein content. Therefore, the fact that MHC I skinned fibre mean myosin content was similar between the experimental groups in the present study suggests that the number of available acto-myosin cross-bridges would have also been similar between groups. The non-significant increase in specific force from solution A to solution B which was greater in young participants compared to both elderly groups (Figure 5.6), is therefore unlikely to be due to a higher total number of available cross-bridges in skinned fibres from young people.

Assuming that the total number of available cross-bridges is similar across the three groups tested in the present study, the trends in the relative force between solutions A and B in each experimental group are likely due to differences in cross-bridge behaviour. Indeed, the hypothesis that cross-bridge behaviour, as opposed to the number of cross-bridges, can modulate skinned fibre specific force has been supported by observations in humans. In spite of reduced myofibrillar protein content, an increased proportion of the available cross-bridges in a bound state during an isometric contraction, maintained MHC IIA Vastus Lateralis specific force in heart failure patients relative to age-matched controls (Miller et al., 2009). Therefore, the greater increase in specific force from solution A to B in young individuals compared to elderly groups may be due to a higher proportion of the available cross-bridges bound during an isometric contraction. Alternatively, previous studies have attributed specific force loss in elderly men (Ochala et al., 2007) and rats (Lowe et al., 2001) to a higher number of acto-myosin cross-bridges

in a low-force producing state. Therefore, the lower increase in specific force in solution B compared to solution A produced by the skinned fibres from elderly cohorts in the present study could be due to a higher number of cross-bridges in a low-force state. However, the lack of statistical significance in the ratio of specific force in solution B to solution A (Figure 5.6) between the different cohorts in the present study suggests that perhaps the differences in skinned fibre responses between groups were not physiologically significant.

5.4.3.2 The potential implications of the X-ray diffraction results

The MM1 X-ray diffraction intensity recordings presented in this study were obtained from sets of approximately thirty skinned fibres undergoing maximal activating contractions in solution A. X-ray diffraction experiments in humans are sparse (Li et al., 2015, Ochala et al., 2011), making this a novel aspect of the present study. The increased intensity of the skinned fibre MM1 reflection, which represents the array of myosin filament heads spaced 14.3nm apart, during contraction in solution A compared to relaxation has been previously observed in human (Li et al., 2015) and mammalian skeletal muscle (Haselgrove, 1975, Huxley et al., 1982, Kraft et al., 2002). The increased MM1 reflection intensity during contraction therefore suggests that a higher number of myosin filament heads are in a bound state during contraction compared to relaxation, which is a logical assumption.

A previous investigation which recorded the MM1 reflection intensity observed a higher degree of disorder in skinned fibres from elderly compared to young individuals (Li et al., 2015). In contrast, in the present study the MM1 reflection intensity during contraction did not differ between the cohorts studied, implying that the number of bound cross-bridges was not different between skinned fibres from all groups contracting in

solution A. However, MM1 reflection intensities from the present study are consistent with the specific force results obtained using skinned fibres from the same participants in solution A. This therefore supports previous observations that human skinned fibre specific force is dependent on the fibre stiffness (Ochala and Larsson, 2008).

Due to the limited amount of human tissue and high number of samples required for X-ray diffraction, the MM1 reflection intensities could not be measured using fibres contracting in solution B. However, the difference in the degree of mean specific force increase in fibres contracting in solution A compared to solution B between the experimental groups studied (Figure 5.6), suggests that the MM1 reflection intensity from fibres contracting in solution B could be increased in young individuals compared to both elderly cohorts. This would imply a difference in the order of the array of myosin filament heads in skinned fibres from young compared to elderly groups and is an experiment for future work.

5.4.4 Inclusion criteria for skinned fibre data

An important point to consider with regard to the data presented in this study is the inclusion criteria utilised. Data were included if the coefficient of variation between a pair of contractions was 28% or less. 28% was selected because it was the value which allowed the inclusion of as many data points as possible without affecting the correlation (R^2) between the mean specific force of a pair of contractions from a skinned fibre in solution A vs solution B. However, perhaps a more appropriate inclusion criteria would be to calculate a threshold for each individual participant included in the present study, as opposed to applying the same threshold across different individuals. This could potentially improve the accuracy with which skinned fibre specific force values are

reflective of a given individual, and could therefore potentially aid in revealing any significant trends, such as that observed in solution B (Figure 5.5).

5.4.5 Summary and conclusions

The present study's findings support that skinned fibre specific force is modulated by myosin content. The difference in the relative increase in specific force in solution B compared to solution A between groups was not statistically significant, although this may be due to a lack of statistical power. Methodological factors such as the activating solutions used to elicit maximal isometric contractions from skinned fibres are likely to contribute to the equivocal results previously reported as to the occurrence of age-related specific force loss at the myofilament level. Furthermore, the observation that myosin loss seems to occur in some (D'Antona et al., 2003) but not all elderly cohorts (Trappe et al., 2003), irrespective of their daily level of physical activity, suggests that identifying individuals susceptible to degradation of myofibrillar protein could be an important predictor of age-induced specific force loss at the skinned fibre level.

6 General Discussion

6.1 General Overview

The initial aim of the present thesis was to assess the impact of physical activity on age-related specific force loss in human skinned muscle fibres, in relation to myosin protein content, cross-bridge behaviour, and positioning of the myosin head during contraction. However, prior to addressing this question, it became clear that there remained unanswered questions regarding the skinned fibre technique. Therefore, before work on human ageing was undertaken, investigations were carried out in order to identify methodological factors which could explain the high degree of variability in published specific force values.

6.2 Systematic review: addressing the variance in published specific force values

The measurement of specific force *in vivo* is complicated by a number of factors which must be accounted for with regard to measuring both P_0 and PCSA. These are offset by using the chemically skinned fibre technique, which enables the study of a single skeletal muscle fibres *in vitro*. However, published skinned fibre specific force values vary greatly between studies, which contradicts the concept that a reliable experimental technique will yield consistent results across different laboratories, even allowing for the lower temperature at which skinned fibre experiments are conducted. Therefore, a systematic review of the skinned fibre literature was undertaken in order to assess the variability in published specific force results, and where possible, to identify factors causing the variability and quantify their effect. The main findings were that:

1. A high degree of greater than 8-fold variability was observed in specific force measured from fibres from the same population (i.e. young, healthy individuals), of the same MHC isoform expression, studied at the same temperature.
2. The ratio of MHC IIA:MHC I fibre specific force was found to be consistent across 62 studies which provided data for both fibre types.
3. When 73 publications were grouped based on shared authorship, 4 main publication groups were observed.
4. The published specific force data was relatively consistent *within* publication groups, compared to *between* groups.
5. The specific force data as published were adjusted for three methodological factors (skinned fibre swelling, CSA measurement technique and sarcomere length) which accounted for 30% of the observed variability in published specific force values.

6.3 The impact of two commonly used activating solutions on the skinned fibre contractile response

The ability to control the experimental solutions surrounding a skinned fibre is an advantage of the technique. However, the activating solutions used to elicit force from skinned skeletal muscle fibres differ between groups of researchers, indicating that this could be a potential source of the variation in skinned fibre specific force values. This methodological difference could not be assessed using systematic review methodology. Therefore, experiments were carried out in order to quantify the effect of different activating solutions, one used by research groups which study human skinned fibres (Solution A) (Larsson and Moss, 1993, Trappe et al., 2003) and one used by local collaborators (solution B) (West et al., 2013), on skinned fibre force, and identify the cause of any observed differences. The main findings were that:

1. The contractile response of the same skinned fibres differed considerably depending on the activating solutions used. Specific force was significantly higher and time to half peak tension (t_{50}) was significantly faster when fibres contracted in solution B.
2. Specific force was significantly lower in an activating solution containing Imidazole, a pH buffer.
3. A lower concentration of Cl^- and the inclusion of glutathione were both individually found to be associated with a faster t_{50}.

6.4 The impact of physical activity on the development of age-related specific force loss in human skinned fibres

Having identified and probed differences between activating solutions, a different approach was taken towards investigating age-related specific force loss using skinned fibres. A three pronged approach was taken which: 1) Assessed specific force in two different activating solutions to identify differential responses to the two solutions. 2) Assessed the relationship between specific force and myosin content from the same skinned fibres. 3) Determine whether differences in the position of the myosin heads during contraction could account for any observed trends in specific force, using low-angle X-ray diffraction experiments. These parameters were measured in skinned fibres from mixed sex cohorts of young, healthy individuals compared to comparatively frail hip fracture patients and physically active master cyclists who had been carefully characterised as part of previous work (Pollock et al., 2015). The hypothesis was that specific force loss would occur in the more frail hip fracture patients and not in the highly active cyclists. The main findings were that:

1. Physical activity did not affect the onset of specific-force loss at the myofilament level in elderly compared to young individuals.
2. Skinned fibre specific force was proportionate to myosin content.
3. The order of the array of myosin heads during contraction was maintained in skinned fibres from elderly individuals, when made to contract in solution A.
4. Cross-bridge behaviour, as defined by the difference in specific force from the same fibres which contracted in solutions A and B, was not significantly different between young and elderly cohorts, although this may be due to a low statistical power.

The relevance of the findings from the aforementioned studies, both with regard to methodological factors associated with the chemically skinned fibre technique and to the physiology of age-related specific force loss, will now be broadly discussed.

The division of the majority of the published, human skinned fibre research into four publication groups is a novel observation. More importantly, the relative consistency of the data within each publication group, compared to across all four groups, supports that methodological differences are partially responsible for the dispersion of the data. That 30% of the variability is caused by methodological factors supports this, but also highlights that the effect of other factors, both methodological and physiological, on the dispersion of the data have not been uncovered. The findings of the systematic review presented in Chapter 2 could benefit the skinned fibre field in the long-term by encouraging efforts to better understand which factors influence skinned fibre contraction which are not physiological, but methodological.

That the skinned fibre contractile response is sensitive to the activating solutions used to elicit a contraction is clearly demonstrated in Chapter 4. Therefore, chemical

differences in activating solutions between publication groups are a likely source of the variance in published specific force values. One way of comprehensively determining the effect of the experimental solutions used by the different research groups on skinned fibre specific force and other contractile measurements would be via a multi-group collaborative study. Each publication group could send frozen aliquots of their solutions to one lab where their effects on the contractile response of skinned fibres could be compared using skinned fibres from the same biopsy sample. This would ensure that a consistent method is used, thereby isolating the effects of the different activating solutions on skinned fibre contraction. Furthermore, the differences in contractile response observed in the different activating solutions used in Chapter 4 suggest that other measurements of cross-bridge kinetics or stiffness are affected by the chemical composition of the solutions used.

The findings presented in Chapter 4 also indicate that the optimal chemical compositions of the activating solutions used to study skinned fibres has not been determined. Initially, testing different pH buffers would be an obvious candidate to assess factors affecting force, since different pH buffers were found to have an effect on force in the present thesis. Logically, optimising the activating solutions used to study skinned skeletal muscle fibres would increase the probability that any observed differences in specific force at the myofilament level would be due to physiological differences between young or elderly cohorts, as opposed to methodological factors.

The findings from Chapter 5 indicate that neither physical activity, nor age, affect specific force at the myofilament level. This observation was supported by measurements of skinned fibre myosin content and findings from preliminary X-ray diffraction experiments, that the order of the myosin heads during contraction was similar between all three groups. The association between specific force and myofibrillar protein content

was consistent with observations from previous studies (D'Antona et al., 2003, Trappe et al., 2003). The relatively consistent relationship observed between myofibrillar protein content and specific force contrasts with the equivocal observations regarding whether specific force loss occurs with advancing age. This suggests that supporting specific force results with data regarding skinned fibre myofibrillar protein content is critical for demonstrating a physiological basis for a given observed difference, or lack of difference, in specific force between different young and elderly cohorts.

The significantly greater specific force in solution B compared to solution A in all groups, directly demonstrated a difference in cross-bridge behaviour between the two solutions, within groups. A non-significant trend was observed for the difference in specific force elicited by solution B compared to solution A to be greater in young than elderly groups. The lack of statistical significance may be due to a lack of statistical power, which if increased, may directly reveal a difference in cross-bridge behaviour between skinned fibres from young and elderly individuals.

6.5 Summary and conclusions

To conclude, the work in the present thesis has demonstrated that differences in published specific force values stem largely from the methodological practices of different publication groups. This variability is also likely to be contributed to by the experimental solutions used to elicit force from skinned skeletal muscle fibres. The work investigating the methodological factors affecting published skinned fibre specific force results is relevant to scientists using the skinned fibre technique in numerous contexts.

The fact that physical activity was found to have no effect on specific force at the level of the myofilaments what somewhat surprising. That specific force was related to myosin content, suggests that myosin content can be maintained in skinned fibres of elderly

individuals. Taken altogether, the data are in general agreement with work from Trappe et al. (2003) and consistent with the mechanism proposed by D'Antona et al. (2003) regarding the relationship between skinned fibre specific force and myosin content.

7 References

ANDREWS, M. A. W., MAUGHAN, D. W., NOSEK, T. M. & GODT, R. E. 1991. Ion-specific and general ionic effects on contraction of skinned fast- twitch skeletal muscle from the rabbit. *Journal of General Physiology,* 98**,** 1105-1125.

ANIANSSON, A., HEDBERG, M., HENNING, G. B. & GRIMBY, G. 1986. Muscle morphology, enzymatic activity, and muscle strength in elderly men: a follow-up study. *Muscle Nerve,* 9**,** 585-91.

APRIL, E. W. & BRANDT, P. W. 1973. The Myofilament Lattice: Studies on Isolated Fibers III. The effect of myofilament spacing upon tension. *The Journal of general physiology,* 61**,** 490-508.

APRIL, E. W., BRANDT, P. W. & ELLIOTT, G. F. 1972. The myofilament lattice: studies on isolated fibers. II. The effects of osmotic strength, ionic concentration, and pH upon the unit-cell volume. *Journal of Cell Biology,* 53**,** 53-65.

ASHLEY, C. & MOISESCU, D. 1977. Effect of changing the composition of the bathing solution upon the isometric tension—pCa relationship in bundles of crustacean myofibrils. *The Journal of physiology,* 270**,** 627-652.

BAMMAN, M. M., CLARKE, M. S., TALMADGE, R. J. & FEEBACK, D. L. 1999. Enhanced protein electrophoresis technique for separating human skeletal muscle myosin heavy chain isoforms. *Electrophoresis,* 20**,** 466-468.

BÁRÁNY, M. 1967. ATPase activity of myosin correlated with speed of muscle shortening. *The Journal of general physiology,* 50**,** 197-218.

BASSEY, E. J. 1998. Longitudinal changes in selected physical capabilities: muscle strength, flexibility and body size. *Age Ageing,* 27 Suppl 3**,** 12-6.

BAUMGARTNER, R. N., WATERS, D. L., GALLAGHER, D., MORLEY, J. E. & GARRY, P. J. 1999. Predictors of skeletal muscle mass in elderly men and women. *Mech Ageing Dev,* 107**,** 123-36.

BIANCHI, C. P. & SHANES, A. 1959. Calcium influx in skeletal muscle at rest, during activity, and during potassium contracture. *The Journal of General Physiology,* 42**,** 803-815.

BLAAUW, B., CANATO, M., AGATEA, L., TONIOLO, L., MAMMUCARI, C., MASIERO, E., ABRAHAM, R., SANDRI, M., SCHIAFFINO, S. & REGGIANI, C. 2009. Inducible activation of Akt increases skeletal muscle mass and force without satellite cell activation. *The FASEB journal,* 23**,** 3896-3905.

BLAIR, S. N., LAMONTE, M. J. & NICHAMAN, M. Z. 2004. The evolution of physical activity recommendations: how much is enough? *Am J Clin Nutr,* 79**,** 913S-920S.

BLOUGH, E. R., RENNIE, E. R., ZHANG, F. & REISER, P. J. 1996. Enhanced electrophoretic separation and resolution of myosin heavy chains in mammalian and avian skeletal muscles. *Analytical biochemistry,* 233**,** 31-35.

BOOTH, F. W. & LEES, S. J. 2006. Physically active subjects should be the control group. *Med Sci Sports Exerc,* 38**,** 405-6.

BOTTINELLI, R., CANEPARI, M., PELLEGRINO, M. & REGGIANI, C. 1996. Force-velocity properties of human skeletal muscle fibres: myosin heavy chain isoform and temperature dependence. *The Journal of physiology,* 495**,** 573-586.

BOWEN, W. J. 1965. Effects of pH, buffers, carnosine, histidine, and β-alanine on the shortening of glycerol-treated muscle fibers. *Archives of biochemistry and biophysics,* 112**,** 436-442.

BRENNER, B., HAHN, N., HANKE, E., MATINMEHR, F., SCHOLZ, T., STEFFEN, W. & KRAFT, T. 2012. Mechanical and kinetic properties of β-cardiac/slow skeletal muscle myosin. *Journal of muscle research and cell motility,* 33**,** 403-417.

BROOKE, M. H. & KAISER, K. K. 1970. Three" myosin adenosine triphosphatase" systems: the nature of their pH lability and sulfhydryl dependence. *Journal of Histochemistry & Cytochemistry,* 18**,** 670-672.

BRUCE, S., NEWTON, D. & WOLEDGE, R. 1989a. Effect of subnutrition on normalized muscle force and relaxation rate in human subjects using voluntary contractions. *Clinical Science,* 76**,** 637-641.

BRUCE, S. A., NEWTON, D. & WOLEDGE, R. C. 1989b. EFFECT OF AGE ON VOLUNTARY FORCE AND CROSS-SECTIONAL AREA OF HUMAN ADDUCTOR POLLICIS MUSCLE. *Quarterly Journal of Experimental Physiology and Cognate Medical Sciences,* 74**,** 359-362.

C. HARRIS, R., DUNNETT, M. & GREENHAFF, P. L. 1998. Carnosine and taurine contents in individual fibres of human vastus lateralis muscle. *Journal of Sports Sciences,* 16**,** 639-643.

CALEY, M. & SIDHU, K. 2011. Estimating the future healthcare costs of an aging population in the UK: expansion of morbidity and the need for preventative care. *Journal of Public Health,* 33**,** 117-122.

CALLAHAN, D. M., MILLER, M. S., SWEENY, A. P., TOURVILLE, T. W., SLAUTERBECK, J. R., SAVAGE, P. D., MAUGAN, D. W., ADES, P. A., BEYNNON, B. D. & TOTH, M. J. 2014. Muscle disuse alters skeletal muscle contractile function at the molecular and cellular levels in older adult humans in a sex-specific manner. *The Journal of physiology,* 592**,** 4555-4573.

CANEPARI, M., ROSSI, R., PELLEGRINO, M., ORRELL, R., COBBOLD, M., HARRIDGE, S. & BOTTINELLI, R. 2005. Effects of resistance training on myosin function studied by the in vitro motility assay in young and older men. *Journal of applied Physiology,* 98**,** 2390-2395.

CANNON, J., TARPENNING, K., KAY, D. & MARINO, F. E. 2001. Ageing is not associated with a decline in neuromuscular innervation or reduced specific force in men aged 20 and 50 years. *Clin Physiol,* 21**,** 350-7.

CRISTEA, A., QAISAR, R., EDLUND, P. K., LINDBLAD, J., BENGTSSON, E. & LARSSON, L. 2010. Effects of aging and gender on the spatial organization of nuclei in single human skeletal muscle cells. *Aging cell,* 9**,** 685-697.

CRUZ-JENTOFT, A. J., BAEYENS, J. P., BAUER, J. M., BOIRIE, Y., CEDERHOLM, T., LANDI, F., MARTIN, F. C., MICHEL, J. P., ROLLAND, Y., SCHNEIDER, S. M., TOPINKOVA, E., VANDEWOUDE, M., ZAMBONI, M. & EUROPEAN WORKING GROUP ON SARCOPENIA IN OLDER, P. 2010. Sarcopenia: European consensus on definition and diagnosis: Report of the European Working Group on Sarcopenia in Older People. *Age Ageing,* 39**,** 412-23.

CURTIN, N., KOMETANI, K. & WOLEDGE, R. 1988. Effect of intracellular pH on force and heat production in isometric contraction of frog muscle fibres. *The Journal of physiology,* 396**,** 93.

CURTIN, N. A., DIACK, R. A., WEST, T. G., WILSON, A. M. & WOLEDGE, R. C. 2015. Skinned fibres produce the same power and force as intact fibre bundles from muscle of wild rabbits. *Journal of Experimental Biology,* 218**,** 2856-2863.

CUTHBERTSON, D., SMITH, K., BABRAJ, J., LEESE, G., WADDELL, T., ATHERTON, P., WACKERHAGE, H., TAYLOR, P. M. & RENNIE, M. J. 2005. Anabolic signaling deficits underlie amino acid resistance of wasting, aging muscle. *The FASEB Journal,* 19**,** 422-424.

D'ANTONA, G., PELLEGRINO, M. A., ADAMI, R., ROSSI, R., NACCARI CARLIZZI, C., CANEPARI, M., SALTIN, B. & BOTTINELLI, R. 2003. The effect of ageing and immobilization on structure and function of human skeletal muscle fibres. *Journal of Physiology,* 552**,** 499-511.

D'ANTONA, G., PELLEGRINO, M. A., CARLIZZI, C. N. & BOTTINELLI, R. 2007. Deterioration of contractile properties of muscle fibres in elderly subjects is modulated by the level of physical activity. *Eur J Appl Physiol,* 100**,** 603-11.

D'ANTONA, G., PELLEGRINO, M. A., CARLIZZI, C. N. & BOTTINELLI, R. 2007. Deterioration of contractile properties of muscle fibres in elderly subjects is modulated by the level of physical activity. *European journal of applied physiology,* 100**,** 603-611.

DAVEY, C. 1960. The significance of carnosine and anserine in striated skeletal muscle. *Archives of Biochemistry and Biophysics,* 89**,** 303-308.

DEGENS, H. & ALWAY, S. E. 2003. Skeletal muscle function and hypertrophy are diminished in old age. *Muscle & nerve,* 27**,** 339-347.

DEGENS, H., HOOFD, L. & BINKHORST, R. 1995. Specific force of the rat plantaris muscle changes with age, but not with overload. *Mechanisms of ageing and development,* 78**,** 215-219.

DEGENS, H. & LARSSON, L. 2007. Application of skinned single muscle fibres to determine myofilament function in ageing and disease. *Journal of Musculoskeletal & Neuronal Interactions,* 7**,** 56-61.

DELMONICO, M. J., HARRIS, T. B., VISSER, M., PARK, S. W., CONROY, M. B., VELASQUEZ-MIEYER, P., BOUDREAU, R., MANINI, T. M., NEVITT, M. & NEWMAN, A. B. 2009. Longitudinal study of muscle strength, quality, and adipose tissue infiltration. *The American journal of clinical nutrition,* 90**,** 1579-1585.

DERAVE, W., ÖZDEMIR, M. S., HARRIS, R. C., POTTIER, A., REYNGOUDT, H., KOPPO, K., WISE, J. A. & ACHTEN, E. 2007. β-Alanine supplementation augments muscle carnosine content and attenuates fatigue during repeated isokinetic contraction bouts in trained sprinters. *Journal of applied physiology,* 103**,** 1736-1743.

DOHERTY, T. J., VANDERVOORT, A. A., TAYLOR, A. W. & BROWN, W. F. 1993. Effects of motor unit losses on strength in older men and women. *Journal of Applied Physiology,* 74**,** 868-874.

DONALDSON, S. K. & KERRICK, W. G. 1975. Characterization of the effects of Mg2+ on Ca2+- and Sr2+-activated tension generation of skinned skeletal muscle fibers. *Journal of General Physiology,* 66**,** 427-44.

EBASHI, F. & EBASHI, S. 1962. Removal of calcium and relaxation in actomyosin systems. *Nature,* 194**,** 378-379.

EDMAN, K. 1979. The velocity of unloaded shortening and its relation to sarcomere length and isometric force in vertebrate muscle fibres. *The Journal of physiology,* 291**,** 143.

ENNION, S., PEREIRA, J. S. A., SARGEANT, A. J., YOUNG, A. & GOLDSPINK, G. 1995. Characterization of human skeletal muscle fibres according to the myosin heavy chains they express. *Journal of Muscle Research & Cell Motility,* 16**,** 35-43.

FABIATO, A. & FABIATO, F. 1978. Effects of pH on the myofilaments and the sarcoplasmic reticulum of skinned cells from cardiace and skeletal muscles. *The Journal of physiology,* 276**,** 233.

FERENCZI, M., GOLDMAN, Y. & SIMMONS, R. 1984. The dependence of force and shortening velocity on substrate concentration in skinned muscle fibres from Rana temporaria. *The Journal of physiology,* 350**,** 519-543.

FILO, R. S., BOHR, D. F. & RUEGG, J. 1965. Glycerinated skeletal and smooth muscle: calcium and magnesium dependence. *Science,* 147**,** 1581-1583.

FINK, R. H., STEPHENSON, D. G. & WILLIAMS, D. A. 1986. Potassium and ionic strength effects on the isometric force of skinned twitch muscle fibres of the rat and toad. *Journal of Physiology,* 370**,** 317-37.

FITTS, R., COSTILL, D. & GARDETTO, P. 1989. Effect of swim exercise training on human muscle fiber function. *Journal of Applied Physiology,* 66**,** 465-475.

FOLDVARI, M., CLARK, M., LAVIOLETTE, L. C., BERNSTEIN, M. A., KALITON, D., CASTANEDA, C., PU, C. T., HAUSDORFF, J. M., FIELDING, R. A. & SINGH, M. A. F. 2000. Association of muscle power with functional status in community-dwelling elderly women. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences,* 55**,** M192-M199.

FRONTERA, W. R., HUGHES, V. A., KRIVICKAS, L. S., KIM, S. K., FOLDVARI, M. & ROUBENOFF, R. 2003. Strength training in older women: Early and late changes in whole muscle and single cells. *Muscle & Nerve,* 28**,** 601-608.

FRONTERA, W. R., HUGHES, V. A., LUTZ, K. J. & EVANS, W. J. 1991. A cross-sectional study of muscle strength and mass in 45- to 78-yr-old men and women. *J Appl Physiol (1985),* 71**,** 644-50.

FRONTERA, W. R. & LARSSON, L. 1997. Contractile studies of single human skeletal muscle fibers: A comparison of different muscles, permeabilization procedures, and storage techniques. *Muscle & Nerve,* 20**,** 948-952.

FRONTERA, W. R., SUH, D., KRIVICKAS, L. S., HUGHES, V. A., GOLDSTEIN, R. & ROUBENOFF, R. 2000a. Skeletal muscle fiber quality in older men and women. *American Journal of Physiology-Cell Physiology,* 279**,** C611-C618.

FRONTERA, W. R., SUH, D., KRIVICKAS, L. S., HUGHES, V. A., GOLDSTEIN, R. & ROUBENOFF, R. 2000b. Skeletal muscle fiber quality in older men and women. *Am J Physiol Cell Physiol,* 279**,** C611-8.

FUCHS, F. & FOX, C. 1982. Parallel measurements of bound calcium and force in glycerinated rabbit psoas muscle fibers. *Biochimica et Biophysica Acta (BBA)-Bioenergetics,* 679**,** 110-115.

GARNER, D. J. & WIDRICK, J. J. 2003. Cross-bridge mechanisms of muscle weakness in multiple sclerosis. *Muscle & nerve,* 27**,** 456-464.

GEEVES, M. A. & GOLDMANN, W. H. 1990. The influence of anions, ionic strength and organic solvents on the interaction between actin and myosin subfragment 1. Portland Press Limited.

GEIGER, P. C., CODY, M. J., MACKEN, R. L. & SIECK, G. C. 2000. Maximum specific force depends on myosin heavy chain content in rat diaphragm muscle fibers. *Journal of applied physiology,* 89**,** 695-703.

GILLIVER, S., DEGENS, H., RITTWEGER, J., SARGEANT, A. & JONES, D. 2009. Variation in the determinants of power of chemically skinned human muscle fibres. *Experimental physiology,* 94**,** 1070-1078.

GODT, R. E. 1974. Calcium-activated tension of skinned muscle fibers of the frog. Dependence on magnesium adenosine triphosphate concentration. *Journal of General Physiology,* 63**,** 722-39.

GODT, R. E. & LINDLEY, B. D. 1982. Influence of temperature upon contractile activation and isometric force production in mechanically skinned muscle fibers of the frog. *The Journal of General Physiology,* 80**,** 279-297.

GODT, R. E. & MAUGHAN, D. W. 1977. Swelling of skinned muscle fibers of the frog. Experimental observations. *Biophysical Journal,* 19**,** 103-16.

GOLLAPUDI, S. K. & LIN, D. C. 2009. Experimental determination of sarcomere force–length relationship in type-I human skeletal muscle fibers. *Journal of biomechanics,* 42**,** 2011-2016.

GOOD, N. E. & IZAWA, S. 1972. [3] Hydrogen ion buffers. *Methods in enzymology,* 24**,** 53-68.

GOOD, N. E., WINGET, G. D., WINTER, W., CONNOLLY, T. N., IZAWA, S. & SINGH, R. M. 1966. Hydrogen ion buffers for biological research*. *Biochemistry,* 5**,** 467-477.

GORDON, A., HUXLEY, A. F. & JULIAN, F. 1966. The variation in isometric tension with sarcomere length in vertebrate muscle fibres. *The Journal of physiology,* 184**,** 170.

GORDON, A. M. & GODT, R. E. 1970. Some effects of hypertonic solutions on contraction and excitation-contraction coupling in frog skeletal muscles. *Journal of General Physiology,* 55**,** 254-75.

GORDON, A. M., GODT, R. E., DONALDSON, S. K. & HARRIS, C. E. 1973. Tension in skinned frog muscle fibers in solutions of varying ionic strength and neutral salt composition. *Journal of General Physiology,* 62**,** 550-74.

GREIG, C., YOUNG, A., SKELTON, D., PIPPET, E., BUTLER, F. & MAHMUD, S. 1994. Exercise studies with elderly volunteers. *Age and Ageing,* 23**,** 185-189.

GREIG, C. A., BOTELLA, J. & YOUNG, A. 1993. The quadriceps strength of healthy elderly people remeasured after eight years. *Muscle Nerve,* 16**,** 6-10.

GROSICKI, G. J., STANDLEY, R. A., MURACH, K. A., RAUE, U., MINCHEV, K., COEN, P. M., NEWMAN, A. B., CUMMINGS, S., HARRIS, T. & KRITCHEVSKY, S. 2016. Improved single muscle fiber quality in the oldest-old. *Journal of Applied Physiology,* 121**,** 878-884.

HARBER, M. & TRAPPE, S. 2008. Single muscle fiber contractile properties of young competitive distance runners. *Journal of Applied Physiology,* 105**,** 629-636.

HARBER, M. P., GALLAGHER, P. M., CREER, A. R., MINCHEV, K. M. & TRAPPE, S. W. 2004. Single muscle fiber contractile properties during a competitive season in male runners. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology,* 287**,** R1124-R1131.

HARPER, S. 2014. Economic and social implications of aging societies. *Science,* 346**,** 587-591.

HARRIDGE, S. D. & LAZARUS, N. R. 2017. Physical activity, aging, and physiological function. *Physiology,* 32**,** 152-161.

HARRIDGE, S. D. R., BOTTINELLI, R., CANEPARI, M., PELLEGRINO, M. A., REGGIANI, C., ESBJORNSSON, M. & SALTIN, B. 1996. Whole-muscle and single-fibre contractile properties and myosin heavy chain isoforms in humans. *Pflugers Archiv-European Journal of Physiology,* 432**,** 913-920.

HASELGROVE, J. 1975. X-ray evidence for conformational changes in the myosin filaments of vertebrate striated muscle. *Journal of molecular biology,* 92**,** 113IN3121-120IN9143.

HAXTON, H. 1944. Absolute muscle force in the ankle flexors of man. *The Journal of physiology,* 103**,** 267-273.

HEYMSFIELD, S. B., ARTEAGA, C., MCMANUS, C., SMITH, J. & MOFFITT, S. 1983. Measurement of muscle mass in humans: validity of the 24-hour urinary creatinine method. *Am J Clin Nutr,* 37**,** 478-94.

HILL, A. V. 1938. The heat of shortening and the dynamic constants of muscle. *Proceedings of the Royal Society of London B: Biological Sciences,* 126**,** 136-195.

HOOK, P., SRIRAMOJU, V. & LARSSON, L. 2001. Effects of aging on actin sliding speed on myosin from single skeletal muscle cells of mice, rats, and humans. *American Journal of Physiology-Cell Physiology,* 280**,** C782-C788.

HUXLEY, A. F. 1957. Muscle structure and theories of contraction. *Prog. Biophys. Biophys. Chem,* 7**,** 255-318.

HUXLEY, A. F. & SIMMONS, R. M. 1971. Proposed mechanism of force generation in striated muscle. *Nature,* 233**,** 533-538.

HUXLEY, H. & BROWN, W. 1967. The low-angle x-ray diagram of vertebrate striated muscle and its behaviour during contraction and rigor. *Journal of molecular biology,* 30**,** 383IN5-434IN16.

HUXLEY, H., FARUQI, A., KRESS, M., BORDAS, J. & KOCH, M. 1982. Time-resolved X-ray diffraction studies of the myosin layer-line reflections during muscle contraction. *Journal of molecular biology,* 158**,** 637-684.

HVID, L. G., ORTENBLAD, N., AAGAARD, P., KJAER, M. & SUETTA, C. 2011. Effects of ageing on single muscle fibre contractile function following short-term immobilisation. *Journal of Physiology-London,* 589**,** 4745-4757.

HVID, L. G., SUETTA, C., AAGAARD, P., KJAER, M., FRANDSEN, U. & ORTENBLAD, N. 2013. Four days of muscle disuse impairs single fiber contractile function in young and old healthy men. *Experimental Gerontology,* 48**,** 154-161.

IWAMOTO, H. 2000. Influence of ionic strength on the actomyosin reaction steps in contracting skeletal muscle fibers. *Biophysical Journal,* 78**,** 3138-49.

JANSSEN, I., HEYMSFIELD, S. B., WANG, Z. M. & ROSS, R. 2000. Skeletal muscle mass and distribution in 468 men and women aged 18-88 yr. *J Appl Physiol (1985),* 89**,** 81-8.

JUBRIAS, S. A., ODDERSON, I. R., ESSELMAN, P. C. & CONLEY, K. E. 1997a. Decline in isokinetic force with age: muscle cross-sectional area and specific force. *Pflugers Arch,* 434**,** 246-53.

JUBRIAS, S. A., ODDERSON, I. R., ESSELMAN, P. C. & CONLEY, K. E. 1997b. Decline in isokinetic force with age: Muscle cross-sectional area and specific force. *Pflugers Archiv-European Journal of Physiology,* 434**,** 246-253.

KALLMAN, D. A., PLATO, C. C. & TOBIN, J. D. 1990. The role of muscle loss in the age-related decline of grip strength: cross-sectional and longitudinal perspectives. *J Gerontol,* 45**,** M82-8.

KARATZAFERI, C., CHINN, M. K. & COOKE, R. 2004. The force exerted by a muscle cross-bridge depends directly on the strength of the actomyosin bond. *Biophysical journal,* 87**,** 2532-2544.

KAWAI, M., WRAY, J. S. & GUTH, K. 1990. Effect of ionic strength on crossbridge kinetics as studied by sinusoidal analysis, ATP hydrolysis rate and X-ray diffraction techniques in chemically skinned rabbit psoas fibres. *Journal of Muscle Research & Cell Motility,* 11**,** 392-402.

KAWAKAMI, Y., ABE, T. & FUKUNAGA, T. 1993. Muscle-fiber pennation angles are greater in hypertrophied than in normal muscles. *Journal of Applied Physiology,* 74**,** 2740-2744.

KENT-BRAUN, J. A. & NG, A. V. 1999. Specific strength and voluntary muscle activation in young and elderly women and men. *J Appl Physiol (1985),* 87**,** 22-9.

KLEIN, C. S., RICE, C. L. & MARSH, G. D. 2001. Normalized force, activation, and coactivation in the arm muscles of young and old men. *Journal of Applied Physiology,* 91**,** 1341-1349.

KORHONEN, M. T., CRISTEA, A., ALÉN, M., HÄKKINEN, K., SIPILÄ, S., MERO, A., VIITASALO, J. T., LARSSON, L. & SUOMINEN, H. 2006. Aging, muscle fiber type, and contractile function in sprint-trained athletes. *Journal of Applied Physiology,* 101**,** 906-917.

KRAFT, T., MATTEI, T., RADOCAJ, A., PIEP, B., NOCULA, C., FURCH, M. & BRENNER, B. 2002. Structural features of cross-bridges in isometrically contracting skeletal muscle. *Biophysical journal,* 82**,** 2536-2547.

KRIVICKAS, L. S., DORER, D. J., OCHALA, J. & FRONTERA, W. R. 2011. Relationship between force and size in human single muscle fibres. *Experimental Physiology,* 96**,** 539-547.

KUMAR, V., ATHERTON, P. J., SELBY, A., RANKIN, D., WILLIAMS, J., SMITH, K., HISCOCK, N. & RENNIE, M. J. 2012. Muscle protein synthetic responses to exercise: effects of age, volume, and intensity. *Journals of Gerontology Series A-Biological Sciences & Medical Sciences,* 67**,** 1170-7.

KUMAR, V., SELBY, A., RANKIN, D., PATEL, R., ATHERTON, P., HILDEBRANDT, W., WILLIAMS, J., SMITH, K., SEYNNES, O. & HISCOCK, N. 2009. Age-related differences in the dose–response relationship of muscle protein synthesis to resistance exercise in young and old men. *The Journal of physiology,* 587**,** 211-217.

LAEMMLI, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *nature,* 227**,** 680-685.

LARSSON, L., LI, X., BERG, H. E. & FRONTERA, W. R. 1996. Effects of removal of weight-bearing function on contractility and myosin isoform composition in single human skeletal muscle cells. *Pflügers Archiv European Journal of Physiology,* 432**,** 320-328.

LARSSON, L., LI, X. & FRONTERA, W. R. 1997. Effects of aging on shortening velocity and myosin isoform composition in single human skeletal muscle cells. *Am J Physiol,* 272**,** C638-49.

LARSSON, L. & MOSS, R. L. 1993. Maximum velocity of shortening in relation to myosin isoform composition in single fibres from human skeletal muscles. *Journal of Physiology,* 472**,** 595-614.

LAZARUS, N. R. & HARRIDGE, S. D. 2010. Exercise, physiological function, and the selection of participants for aging research. *J Gerontol A Biol Sci Med Sci,* 65**,** 854-7.

LAZARUS, N. R. & HARRIDGE, S. D. 2016. Declining performance of master athletes: Silhouettes of the trajectory of healthy human ageing? *The Journal of Physiology*.

LEXELL, J., DOWNHAM, D. & SJOSTROM, M. 1983. Distribution of different fibre types in human skeletal muscles. A statistical and computational model for the study of fibre type grouping and early diagnosis of skeletal muscle fibre denervation and reinnervation. *J Neurol Sci,* 61**,** 301-14.

LEXELL, J., DOWNHAM, D. & SJOSTROM, M. 1986. Distribution of different fibre types in human skeletal muscles. Fibre type arrangement in m. vastus lateralis from three groups of healthy men between 15 and 83 years. *J Neurol Sci,* 72**,** 211-22.

LEXELL, J., TAYLOR, C. C. & SJOSTROM, M. 1988. What is the cause of the ageing atrophy? Total number, size and proportion of different fiber types studied in whole vastus lateralis muscle from 15- to 83-year-old men. *J Neurol Sci,* 84**,** 275-94.

LI, M., OGILVIE, H., OCHALA, J., ARTEMENKO, K., IWAMOTO, H., YAGI, N., BERGQUIST, J. & LARSSON, L. 2015. Aberrant post-translational modifications compromise human myosin motor function in old age. *Aging Cell,* 14**,** 228-235.

LINDLE, R. S., METTER, E. J., LYNCH, N. A., FLEG, J. L., FOZARD, J. L., TOBIN, J., ROY, T. A. & HURLEY, B. F. 1997. Age and gender comparisons of muscle strength in 654 women and men aged 20-93 yr. *J Appl Physiol (1985),* 83**,** 1581-7.

LOWE, D. A., SUREK, J. T., THOMAS, D. D. & THOMPSON, L. V. 2001. Electron paramagnetic resonance reveals age-related myosin structural changes in rat skeletal muscle fibers. *American Journal of Physiology-Cell Physiology,* 280**,** C540-C547.

MACALUSO, A., NIMMO, M. A., FOSTER, J. E., COCKBURN, M., MCMILLAN, N. C. & DE VITO, G. 2002. Contractile muscle volume and agonist-antagonist coactivation account for differences in torque between young and older women. *Muscle & Nerve,* 25**,** 858-863.

MADSEN, O. R., LAURIDSEN, U. B., HARTKOPP, A. & SORENSEN, O. H. 1997. Muscle strength and soft tissue composition as measured by dual energy x-ray absorptiometry in women aged 18-87 years. *Eur J Appl Physiol Occup Physiol,* 75**,** 239-45.

MATSUBARA, I. & ELLIOTT, G. F. 1972. X-ray diffraction studies on skinned single fibres of frog skeletal muscle. *Journal of molecular biology,* 72**,** 657-669.

MEIJER, J., JASPERS, R., RITTWEGER, J., SEYNNES, O. R., KAMANDULIS, S., BRAZAITIS, M., SKURVYDAS, A., PIŠOT, R., ŠIMUNIČ, B. & NARICI, M. V. 2015. Single muscle fibre contractile properties differ between body-builders, power athletes and control subjects. *Experimental physiology,* 100**,** 1331-1341.

MEN, Y., YOUNG, A., STOKES, M. & CROWE, M. 1985. The size and strength of the quadriceps muscles of old. *Clinical Physiology and Functional Imaging,* 5**,** 145-154.

METZGER, J. & MOSS, R. EFFECTS ON TENSION AND STIFFNESS DUE TO REDUCED PH IN ISOLATED RABBIT FAST-TWITCH AND SLOW-TWITCH SINGLE SKELETAL-MUSCLE FIBERS. JOURNAL OF PHYSIOLOGY-LONDON, 1989. CAMBRIDGE UNIV PRESS 40 WEST 20TH STREET, NEW YORK, NY 10011-4211, P67-P67.

MILLER, M. S., BEDRIN, N. G., ADES, P. A., PALMER, B. M. & TOTH, M. J. 2015. Molecular determinants of force production in human skeletal muscle fibers: effects of myosin isoform expression and cross-sectional area. *American Journal of Physiology-Cell Physiology,* 308**,** C473-C484.

MILLER, M. S., BEDRIN, N. G., CALLAHAN, D. M., PREVIS, M. J., JENNINGS, M. E., ADES, P. A., MAUGHAN, D. W., PALMER, B. M. & TOTH, M. J. 2013. Age-related slowing of myosin actin cross-bridge kinetics is sex specific and predicts decrements in whole skeletal muscle performance in humans. *Journal of Applied Physiology,* 115**,** 1004-1014.

MILLER, M. S., CALLAHAN, D. M. & TOTH, M. J. 2014. Skeletal muscle myofilament adaptations to aging, disease, and disuse and their effects on whole muscle performance in older adult humans. *Frontiers in Physiology,* 5.

MILLER, M. S., VANBUREN, P., LEWINTER, M. M., LECKER, S. H., SELBY, D. E., PALMER, B. M., MAUGHAN, D. W., ADES, P. A. & TOTH, M. J. 2009. Mechanisms underlying skeletal muscle weakness in human heart failure: alterations in single fiber myosin protein content and function. *Circulation: Heart Failure***,** CIRCHEARTFAILURE. 109.876433.

MITCHELL, W. K., WILLIAMS, J., ATHERTON, P., LARVIN, M., LUND, J. & NARICI, M. 2015. Sarcopenia, dynapenia, and the impact of advancing age on human skeletal muscle size and strength; a quantitative review. *Physiology and Pathophysiology of Musculoskeletal Aging,* 3**,** 1-18.

MOISESCU, D. G. 1976. Kinetics of reaction in calcium activated skinned muscle fibers. *Nature,* 262**,** 610-613.

MOISESCU, D. G. & THIELECZEK, R. 1978. Calcium and strontium concentration changes within skinned muscle preparations following a change in the external bathing solution. *Journal of Physiology,* Vol. 275**,** 241-262.

MOKDAD, A. H., MARKS, J. S., STROUP, D. F. & GERBERDING, J. L. 2004. Actual causes of death in the United States, 2000. *JAMA,* 291**,** 1238-45.

MORRISSEY, J. H. 1981. Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. *Analytical biochemistry,* 117**,** 307-310.

MORSE, C. I., THOM, J. M., REEVES, N. D., BIRCH, K. M. & NARICI, M. V. 2005. In vivo physiological cross-sectional area and specific force are reduced in the gastrocnemius of elderly men. *Journal of Applied Physiology,* 99**,** 1050-1055.

MOSS, R. 1979. Sarcomere length-tension relations of frog skinned muscle fibres during calcium activation at short lengths. *The Journal of physiology,* 292**,** 177-192.

MÜHLRAD, A., KOVACS, M. & HEGYI, G. 1965. The role of Mg2+ in the contraction and adenosine triphosphatase activity of myofibrils. *Biochimica et Biophysica Acta (BBA)-General Subjects,* 107**,** 567-578.

MURPHY, R., DUTKA, T. & LAMB, G. 2008. Hydroxyl radical and glutathione interactions alter calcium sensitivity and maximum force of the contractile apparatus in rat skeletal muscle fibres. *The Journal of physiology,* 586**,** 2203-2216.

MURPHY, R. & KOSS, P. 1968. Hydrogen ion buffers and enzymatic activity: Myosin B adenosinetriphosphatase. *Archives of biochemistry and biophysics,* 128**,** 236-242.

NARICI, M. V. & MAFFULLI, N. 2010. Sarcopenia: characteristics, mechanisms and functional significance. *British medical bulletin,* 95**,** 139-159.

NARICI, M. V., MAGANARIS, C. N., REEVES, N. D. & CAPODAGLIO, P. 2003. Effect of aging on human muscle architecture. *Journal of applied physiology,* 95**,** 2229-2234.

O'BRIEN, T. D., REEVES, N. D., BALTZOPOULOS, V., JONES, D. A. & MAGANARIS, C. N. 2010. In vivo measurements of muscle specific tension in adults and children. *Experimental Physiology,* 95**,** 202-210.

O'BRIEN, T. D., REEVES, N. D., BALTZOPOULOS, V., JONES, D. A. & MAGANARIS, C. N. 2010. In vivo measurements of muscle specific tension in adults and children. *Experimental physiology,* 95**,** 202-210.

OCHALA, J., FRONTERA, W. R., DORER, D. J., VAN HOECKE, J. & KRIVICKAS, L. S. 2007. Single skeletal muscle fiber elastic and contractile characteristics in young and older men. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences,* 62**,** 375-381.

OCHALA, J. & LARSSON, L. 2008. Effects of a preferential myosin loss on Ca2+ activation of force generation in single human skeletal muscle fibres. *Experimental Physiology,* 93**,** 486-495.

OCHALA, J., LEHTOKARI, V.-L., IWAMOTO, H., LI, M., FENG, H.-Z., JIN, J.-P., YAGI, N., WALLGREN-PETTERSSON, C., PÉNISSON-BESNIER, I. & LARSSON, L. 2011. Disrupted myosin cross-

bridge cycling kinetics triggers muscle weakness in nebulin-related myopathy. *The FASEB Journal,* 25**,** 1903-1913.

OGAWA, Y. 1968. The apparent binding constant of glycoletherdiaminetetraacetic acid for calcium at neutral pH. *The Journal of Biochemistry,* 64**,** 255-257.

ORENTLICHER, M., REUBEN, J., GRUNDFEST, H. & BRANDT, P. 1974. Calcium binding and tension development in detergent-treated muscle fibers. *The Journal of general physiology,* 63**,** 168-186.

OSTAP, E. M., BARNETT, V. A. & THOMAS, D. D. 1995. Resolution of three structural states of spin-labeled myosin in contracting muscle. *Biophysical journal,* 69**,** 177-188.

OVEREND, T. J., CUNNINGHAM, D. A., KRAMER, J. F., LEFCOE, M. S. & PATERSON, D. H. 1992. Knee extensor and knee flexor strength: cross-sectional area ratios in young and elderly men. *J Gerontol,* 47**,** M204-10.

PANSARASA, O., RINALDI, C., PARENTE, V., MIOTTI, D., CAPODAGLIO, P. & BOTTINELLI, R. 2009. Resistance training of long duration modulates force and unloaded shortening velocity of single muscle fibres of young women. *Journal of Electromyography and Kinesiology,* 19**,** e290-e300.

PARENTE, V., D'ANTONA, G., ADAMI, R., MIOTTI, D., CAPODAGLIO, P., DE VITO, G. & BOTTINELLI, R. 2008. Long-term resistance training improves force and unloaded shortening velocity of single muscle fibres of elderly women. *European journal of applied physiology,* 104**,** 885.

PATHARE, N., WALTER, G. A., STEVENS, J. E., YANG, Z. H., OKERKE, E., GIBBS, J. D., ESTERHAI, J. L., SCARBOROUGH, M. T., GIBBS, C. P., SWEENEY, H. L. & VANDENBORNE, K. 2005. Changes in inorganic phosphate and force production in human skeletal muscle after cast immobilization. *Journal of Applied Physiology,* 98**,** 307-314.

PHILLIPS, S. K., BRUCE, S. A., NEWTON, D. & WOLEDGE, R. C. 1992. The weakness of old age is not due to failure of muscle activation. *J Gerontol,* 47**,** M45-9.

PICQUET, F. 2003. Compared effects of hindlimb unloading versus terrestrial deafferentation on muscular properties of the rat soleus. *Experimental Neurology,* 182**,** 186-194.

POLLOCK, R. D., CARTER, S., VELLOSO, C. P., DUGGAL, N. A., LORD, J. M., LAZARUS, N. R. & HARRIDGE, S. D. 2015. An investigation into the relationship between age and physiological function in highly active older adults. *The Journal of physiology,* 593**,** 657-680.

POSTERINO, G. S. & LAMB, G. D. 1996. Effects of reducing agents and oxidants on excitation-contraction coupling in skeletal muscle fibres of rat and toad. *Journal of Physiology,* 496**,** 809-825.

POWER, G. A., MINOZZO, F. C., SPENDIFF, S., FILION, M.-E., KONOKHOVA, Y., PURVES-SMITH, M. F., PION, C., AUBERTIN-LEHEUDRE, M., MORAIS, J. A. & HERZOG, W. 2016. Reduction in single muscle fiber rate of force development with aging is not attenuated in world class older masters athletes. *American Journal of Physiology-Cell Physiology,* 310**,** C318-C327.

PUTHUCHEARY, Z. A., RAWAL, J., MCPHAIL, M., CONNOLLY, B., RATNAYAKE, G., CHAN, P., HOPKINSON, N. S., PHADKE, R., DEW, T. & SIDHU, P. S. 2013. Acute skeletal muscle wasting in critical illness. *Jama,* 310**,** 1591-1600.

RALL, J. A. & WOLEDGE, R. C. 1990. Influence of temperature on mechanics and energetics of muscle contraction. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology,* 259**,** R197-R203.

RAMAMURTHY, B., HÖÖK, P., JONES, A. D. & LARSSON, L. 2001. Changes in myosin structure and function in response to glycation. *The FASEB Journal,* 15**,** 2415-2422.

RANATUNGA, K., SHARPE, B. & TURNBULL, B. 1987. Contractions of a human skeletal muscle at different temperatures. *The Journal of physiology,* 390**,** 383-395.

RANTANEN, T., MASAKI, K., FOLEY, D., IZMIRLIAN, G., WHITE, L. & GURALNIK, J. M. 1998. Grip strength changes over 27 yr in Japanese-American men. *J Appl Physiol (1985),* 85**,** 2047-53.

RANVIER, L. 1873. Propriétés et structures différentes des muscles rouges et des muscles blancs chez les lapins et chez les raies. *CR Acad Sci Paris,* 77**,** 1030-1034.

RAUE, U., SLIVKA, D., MINCHEV, K. & TRAPPE, S. 2009. Improvements in whole muscle and myocellular function are limited with high-intensity resistance training in octogenarian women. *Journal of Applied Physiology,* 106**,** 1611-1617.

REID, K. F., DOROS, G., CLARK, D. J., PATTEN, C., CARABELLO, R. J., CLOUTIER, G. J., PHILLIPS, E. M., KRIVICKAS, L. S., FRONTERA, W. R. & FIELDING, R. A. 2012. Muscle power failure in mobility-limited older adults: preserved single fiber function despite lower whole muscle size, quality and rate of neuromuscular activation. *European Journal of Applied Physiology,* 112**,** 2289-2301.

REID, K. F., PASHA, E., DOROS, G., CLARK, D. J., PATTEN, C., PHILLIPS, E. M., FRONTERA, W. R. & FIELDING, R. A. 2014. Longitudinal decline of lower extremity muscle power in healthy and mobility-limited older adults: influence of muscle mass, strength, composition, neuromuscular activation and single fiber contractile properties. *European Journal of Applied Physiology,* 114**,** 29-39.

RILEY, D. A., BAIN, J. L. W., THOMPSON, J. L., FITTS, R. H., WIDRICK, J. J., TRAPPE, S. W., TRAPPE, T. A. & COSTILL, D. L. 2000. Decreased thin filament density and length in human atrophic soleus muscle fibers after spaceflight. *Journal of Applied Physiology,* 88**,** 567-572.

ROSENBERG, I. H. 1989. Summary comments. *The American Journal of Clinical Nutrition,* 50**,** 1231-1233.

SANT'ANA PEREIRA, J. A. A., WESSELS, A., NIJTMANS, L., MOORMAN, A. F. M. & SARGEANT, A. J. 1995. New method for the accurate characterization of single human skeletal muscle fibres demonstrates a relation between mATPase and MyHC expression in pure and hybrid fibre types. *Journal of Muscle Research and Cell Motility,* 16**,** 21-34.

SCHIAFFINO, S. & REGGIANI, C. 2011. Fiber types in mammalian skeletal muscles. *Physiological reviews,* 91**,** 1447-1531.

SCUFFHAM, P., CHAPLIN, S. & LEGOOD, R. 2003. Incidence and costs of unintentional falls in older people in the United Kingdom. *Journal of epidemiology and community health,* 57**,** 740-744.

SEIDEL, J. C. & GERGELY, J. 1963. Contraction of glycerinated muscle fibers and the role of calcium. *Biochemical and Biophysical Research Communications,* 13**,** 343-347.

SKELTON, D. A., GREIG, C. A., DAVIES, J. M. & YOUNG, A. 1994. Strength, power and related functional ability of healthy people aged 65-89 years. *Age Ageing,* 23**,** 371-7.

SMITH, E. B. 1938. The buffering of muscle in rigor; protein, phosphate and carnosine. *The Journal of physiology,* 92**,** 336.

STARLING, R. D., ADES, P. A. & POEHLMAN, E. T. 1999. Physical activity, protein intake, and appendicular skeletal muscle mass in older men. *Am J Clin Nutr,* 70**,** 91-6.

STEPHENSON, E. W. & PODOLSKY, R. J. 1977. Regulation by magnesium of intracellular calcium movement in skinned muscle fibers. *Journal of General Physiology,* 69**,** 1-16.

STIENEN, G. 2000. Chronicle of skinned muscle fibres. *The Journal of physiology,* 527**,** 1-1.

STIENEN, G. J., KIERS, J. L., BOTTINELLI, R. & REGGIANI, C. 1996. Myofibrillar ATPase activity in skinned human skeletal muscle fibres: fibre type and temperature dependence. *Journal of Physiology,* 493**,** 299-307.

SUGI, H., ABE, T., KOBAYASHI, T., CHAEN, S., OHNUKI, Y., SAEKI, Y. & SUGIURA, S. 2013. Enhancement of force generated by individual myosin heads in skinned rabbit psoas muscle fibers at low ionic strength. *PLoS ONE [Electronic Resource],* 8**,** e63658.

TALMADGE, R. J. & ROY, R. R. 1993. Electrophoretic separation of rat skeletal muscle myosin heavy-chain isoforms. *Journal of Applied Physiology,* 75**,** 2337-2340.

TANAKA, H. & SEALS, D. R. 2003. Invited Review: Dynamic exercise performance in Masters athletes: insight into the effects of primary human aging on physiological functional capacity. *J Appl Physiol (1985),* 95**,** 2152-62.

THOM, J. M., MORSE, C. I., BIRCH, K. M. & NARICI, M. V. 2007. Influence of muscle architecture on the torque and power–velocity characteristics of young and elderly men. *European journal of applied physiology,* 100**,** 613-619.

THOMPSON, L. V., DURAND, D., FUGERE, N. A. & FERRINGTON, D. A. 2006. Myosin and actin expression and oxidation in aging muscle. *J Appl Physiol (1985),* 101**,** 1581-7.

TOTH, M. J., MILLER, M. S., CALLAHAN, D. M., SWEENY, A. P., NUNEZ, I., GRUNBERG, S. M., DER-TOROSSIAN, H., COUCH, M. E. & DITTUS, K. 2013. Molecular mechanisms underlying skeletal muscle weakness in human cancer: reduced myosin-actin cross-bridge formation and kinetics. *Journal of applied physiology,* 114**,** 858-868.

TOTH, M. J., MILLER, M. S., VANBUREN, P., BEDRIN, N. G., LEWINTER, M. M., ADES, P. A. & PALMER, B. M. 2012. Resistance training alters skeletal muscle structure and function in human heart failure: effects at the tissue, cellular and molecular levels. *The Journal of physiology,* 590**,** 1243-1259.

TRAPPE, S., CREER, A., MINCHEV, K., SLIVKA, D., LOUIS, E., LUDEN, N. & TRAPPE, T. 2008. Human soleus single muscle fiber function with exercise or nutrition countermeasures during 60 days of bed rest. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology,* 294**,** R939-R947.

TRAPPE, S., CREER, A., SLIVKA, D., MINCHEV, K. & TRAPPE, T. 2007. Single muscle fiber function with concurrent exercise or nutrition countermeasures during 60 days of bed rest in women. *Journal of Applied Physiology,* 103**,** 1242-1250.

TRAPPE, S., GALLAGHER, P., HARBER, M., CARRITHERS, J., FLUCKEY, J. & TRAPPE, T. 2003. Single muscle fibre contractile properties in young and old men and women. *The Journal of physiology,* 552**,** 47-58.

TRAPPE, S., HARBER, M., CREER, A., GALLAGHER, P., SLIVKA, D., MINCHEV, K. & WHITSETT, D. 2006. Single muscle fiber adaptations with marathon training. *Journal of Applied Physiology,* 101**,** 721-727.

TRAPPE, S., TRAPPE, T., GALLAGHER, P., HARBER, M., ALKNER, B. & TESCH, P. 2004. Human single muscle fibre function with 84 day bed-rest and resistance exercise. *Journal of Physiology-London,* 557**,** 501-513.

TRAPPE, S., WILLIAMSON, D., GODARD, M., PORTER, D., ROWDEN, G. & COSTILL, D. 2000. Effect of resistance training on single muscle fiber contractile function in older men. *Journal of Applied Physiology,* 89**,** 143-152.

VANDERVOORT, A. A. & MCCOMAS, A. J. 1986. Contractile changes in opposing muscles of the human ankle joint with aging. *J Appl Physiol (1985),* 61**,** 361-7.

VENTURELLI, M., SAGGIN, P., MUTI, E., NARO, F., CANCELLARA, L., TONIOLO, L., TARPERI, C., CALABRIA, E., RICHARDSON, R. S., REGGIANI, C. & SCHENA, F. 2015. In vivo and in vitro evidence that intrinsic upper- and lower-limb skeletal muscle function is unaffected by ageing and disuse in oldest-old humans. *Acta Physiologica,* 215**,** 58-71.

VISSER, M., GOODPASTER, B. H., KRITCHEVSKY, S. B., NEWMAN, A. B., NEVITT, M., RUBIN, S. M., SIMONSICK, E. M. & HARRIS, T. B. 2005. Muscle mass, muscle strength, and muscle fat infiltration as predictors of incident mobility limitations in well-functioning older persons. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences,* 60**,** 324-333.

WEST, T. G., FERENCZI, M. A., WOLEDGE, R. C. & CURTIN, N. 2005. Influence of ionic strength on the time course of force development and phosphate release by dogfish muscle fibres. *The Journal of physiology,* 567**,** 989-1000.

WEST, T. G., TOEPFER, C. N., WOLEDGE, R. C., CURTIN, N. A., ROWLERSON, A., KALAKOUTIS, M., HUDSON, P. & WILSON, A. M. 2013. Power output of skinned skeletal muscle fibres from the cheetah (Acinonyx jubatus). *Journal of Experimental Biology,* 216**,** 2974-2982.

WESTERBLAD, H., BRUTON, J. & LÄNNERGREN, J. 1997. The effect of intracellular pH on contractile function of intact, single fibres of mouse muscle declines with increasing temperature. *The Journal of physiology,* 500**,** 193.

WIDRICK, J. J., KNUTH, S. T., NORENBERG, K. M., ROMATOWSKI, J. G., BAIN, J. L. W., RILEY, D. A., KARHANEK, M., TRAPPE, S. W., TRAPPE, T. A., COSTILL, D. L. & FITTS, R. H. 1999. Effect of a 17 day spaceflight on contractile properties of human soleus muscle fibres. *Journal of Physiology-London,* 516**,** 915-930.

WIDRICK, J. J., NORENBERG, K. M., ROMATOWSKI, J., BLASER, C., KARHANEK, M., SHERWOOD, J., TRAPPE, S., TRAPPE, T., COSTILL, D. & FITTS, R. 1998. Force-velocity-power and force-pCa relationships of human soleus fibers after 17 days of bed rest. *Journal of Applied Physiology,* 85**,** 1949-1956.

WIDRICK, J. J., ROMATOWSKI, J. G., BAIN, J. L. W., TRAPPE, S. W., TRAPPE, T. A., THOMPSON, J. L., COSTILL, D. L., RILEY, D. A. & FITTS, R. H. 1997. Effect of 17 days of bed rest on peak isometric force and unloaded shortening velocity of human soleus fibers. *American Journal of Physiology-Cell Physiology,* 273**,** C1690-C1699.

WIDRICK, J. J., ROMATOWSKI, J. G., NORENBERG, K. M., KNUTH, S. T., BAIN, J. L. W., RILEY, D. A., TRAPPE, S. W., TRAPPE, T. A., COSTILL, D. L. & FITTS, R. H. 2001. Functional properties of slow and fast gastrocnemius muscle fibers after a 17-day spaceflight. *Journal of Applied Physiology,* 90**,** 2203-2211.

WIDRICK, J. J., TRAPPE, S. W., BLASER, C. A., COSTILL, D. L. & FITTS, R. H. 1996a. Isometric force and maximal shortening velocity of single muscle fibers from elite master runners. *American Journal of Physiology-Cell Physiology,* 271**,** C666-C675.

WIDRICK, J. J., TRAPPE, S. W., COSTILL, D. L. & FITTS, R. H. 1996b. Force-velocity and force-power properties of single muscle fibers from elite master runners and sedentary men. *American Journal of Physiology-Cell Physiology,* 271**,** C676-C683.

WIDRICK, J. J., TRAPPE, S. W., ROMATOWSKI, J. G., RILEY, D. A., COSTILL, D. L. & FITTS, R. H. 2002. Unilateral lower limb suspension does not mimic bed rest or spaceflight effects on human muscle fiber function. *Journal of Applied Physiology,* 93**,** 354-360.

WINEGARD, K. J., HICKS, A. L., SALE, D. G. & VANDERVOORT, A. A. 1996. A 12-year follow-up study of ankle muscle function in older adults. *J Gerontol A Biol Sci Med Sci,* 51**,** B202-7.

WOLEDGE, R., BARCLAY, C. J. & CURTIN, N. 2009. Temperature change as a probe of muscle crossbridge kinetics: a review and discussion. *Proceedings of the Royal Society of London B: Biological Sciences,* 276**,** 2685-2695.

WROBLEWSKI, A. P., AMATI, F., SMILEY, M. A., GOODPASTER, B. & WRIGHT, V. 2011. Chronic exercise preserves lean muscle mass in masters athletes. *The Physician and Sportsmedicine,* 39**,** 172-178.

YORK, D., EVENSEN, N. M., MARTıNEZ, M. L. & DELGADO, J. D. B. 2004. Unified equations for the slope, intercept, and standard errors of the best straight line. *American Journal of Physics,* 72**,** 367-375.

YU, F., HEDSTROM, M., CRISTEA, A., DALEN, N. & LARSSON, L. 2007. Effects of ageing and gender on contractile properties in human skeletal muscle and single fibres. *Acta Physiologica,* 190**,** 229-241.

8 Appendices

8.1 Health questionnaire completed by healthy, young participants and hip fracture patients.

Health Questionnaire

Name:

Address:

Date of birth:

Telephone no:

If the answer is YES to any questions please give some details including dates where possible.

1. Have you any history of heart trouble?

(Such as heart attack, angina, valve disease, palpitations, pains in chest, dizzy spells)

_____
_____
_____
_____

2. Have you any history of problems with blood vessels?

(Such as thrombosis, embolus, claudication, aneurysm, dizzy spells, stroke, blood clots)

_____
_____
_____
_____

3. Have you any history of chest problems?

(Bronchitis, asthma or wheezy chest)

_____
_____

_____

_____

4. Have you ever smoked?

   (If YES please state whether you are a current or ex-smoker and how much)

_____

_____

_____

_____

5. Do you suffer from diabetes?

   (If YES please state if insulin dependent)

_____

_____

_____

_____

6. Have you any history of major illness now or in the last 20 yrs?

   (Such as rheumatoid arthritis, blood disorders, cancer).

_____

_____

_____

_____

7. Have you any history of emotional or psychiatric problems?

_____

_____

_____

_____

8. Do you suffer from osteoarthritis or rheumatoid arthritis?

   (If YES please state joints affected and indicate mild, moderate or severe and any medication regularly taken).

_____

_____

_____

_____

9. Have you broken or fractured any bones? If so, which bones and when?

_____

_____

_____

_____

10. Do you have any problems with your bones

(Diagnosed osteoporosis, loss of height)

_____

_____

_____

_____

11. Have you any history of back problems?

_____

_____

_____

_____

12. Have you had any surgery on your joints?

_____

_____

_____

_____

13. Do you suffer from high blood pressure?

_____

_____

_____

_____

14. Have you had any acute illness in the last six months?

(Such as influenza, recurrent sore-throat, bronchitis)

_____
_____
_____
_____

15. Please state any medication regularly taken for any condition.

_____
_____
_____
_____

16. Have you been in hospital in the last 5 years and if so, for how long?

_____
_____
_____
_____

17. Do you have any physical disabilities?

    (Such as visual or hearing problems)

_____
_____
_____
_____

18. Do you suffer from Multiple Sclerosis or Parkinson's disease?

_____
_____
_____
_____

19. Is there any other illness or condition that affects your general health or interferes with your mobility?

_____
_____
_____
_____

20. How many times have you fallen in the past year (approximately)?

_____
_____
_____
_____

21. Approximately how tall are you?

_____
_____
_____
_____

22. Approximately how much do you weigh?

_____
_____
_____
_____

Print name: _____

Sign: _____ Date:

_____

8.2 Fibre typing cheetah muscle fibres

The protocol used to differentiate between skinned fibres containing MHC I and MHC II myosin heavy chain isoforms, as seen in Figure 3.3, is outlined here. Fibre segments 1-3mm in length from gluteus, semitendinosus and longissimus muscle of the African cheetah were dissolved in 20µl of sample buffer (Laemmli, 1970) and heated at 100^0C for 3 minutes. Four microlitre samples were loaded into 0.75mm thick gels in a Hoefer 'mighty small' apparatus. A 4.5% stacking gel with a 7.5% separating gel containing 30% glycerol was used (Picquet, 2003). The gels were run using the method of Picquet (2003) with minor modifications. Over a 12 hour period a constant voltage of 100V was set until the bromophenyl blue marker had migrated significantly (~2cm) into the separating gel, at which point the voltage was increased to 180V for the remainder of the run. Gels were subsequently silver stained (Morrissey, 1981) to reveal the myosin heavy chain bands. A sample of gluteus muscle, which contains both MHC I and MHC II fibres, underwent the same treatment as the single fibre segments and was used as a standard. Two clearly separated bands were seen in this standard which were assumed to be from the fast and slow fibres.

8.1 Constituents of SDS PAGE gels used to fibre type skinned skeletal muscle fibres.

Stock solution	Separating Gel (ml)
100% Glycerol	3
30% Acrylamide-bis (50:1)	2.667
Tris 1.5M (pH 8.8)	1.333
1M Glycine	1
10% SDS	0.4
Distilled water	1.495
TEMED	0.005
10% Ammonium persulfate.	0.1
Total volume	10ml

Table 8.1. The constituents and subsequent quantities required to make up the 8% separating gel used to fibre type skinned single skeletal muscle fibres in the present thesis (Talmadge and Roy, 1993).

Stock solution	Stacking Gel (ml)
100% Glycerol	3
30% Acrylamide-bis (50:1)	1.333
Tris 0.5M (pH 6.7)	1.4
100mM EDTA (pH 7.0)	0.4
10% SDS	0.4
Distilled water	3.362
TEMED	0.005
10% Ammonium persulfate	0.100
Total volume	10ml

Table 8.2. The constituents and subsequent quantities required to make up the 4% separating gel used to fibre type skinned single skeletal muscle fibres in the present thesis (Talmadge and Roy, 1993).

Chemical	Upper running buffer	Lower running buffer
Tris	3g	3g
Glycine	14.4g	14.4g
SDS	1g	1g
B-mercaptoethanol	None	1%

Table 8.3. Chemical constituents of upper and lower running buffer used to fibre type skinned skeletal muscle fibres (Blough et al., 1996).

Laemmli sample buffer	
Chemical	Quantity
Tris-HCL pH 6.8	0.91g
4% SDS	2g
20% Glycerol	10ml
10% β-mercaptoethanol	5ml
0.002% bromophenol blue	1mg
Distilled water	82ml
Total volume	100ml

Table 8.4. Ingredients and quantities to make up 100ml of Laemmli sample buffer (Laemmli, 1970), for preparation of skinned fibre samples for SDS PAGE.

8.2 Forest plots of the variance in published skinned fibre specific force

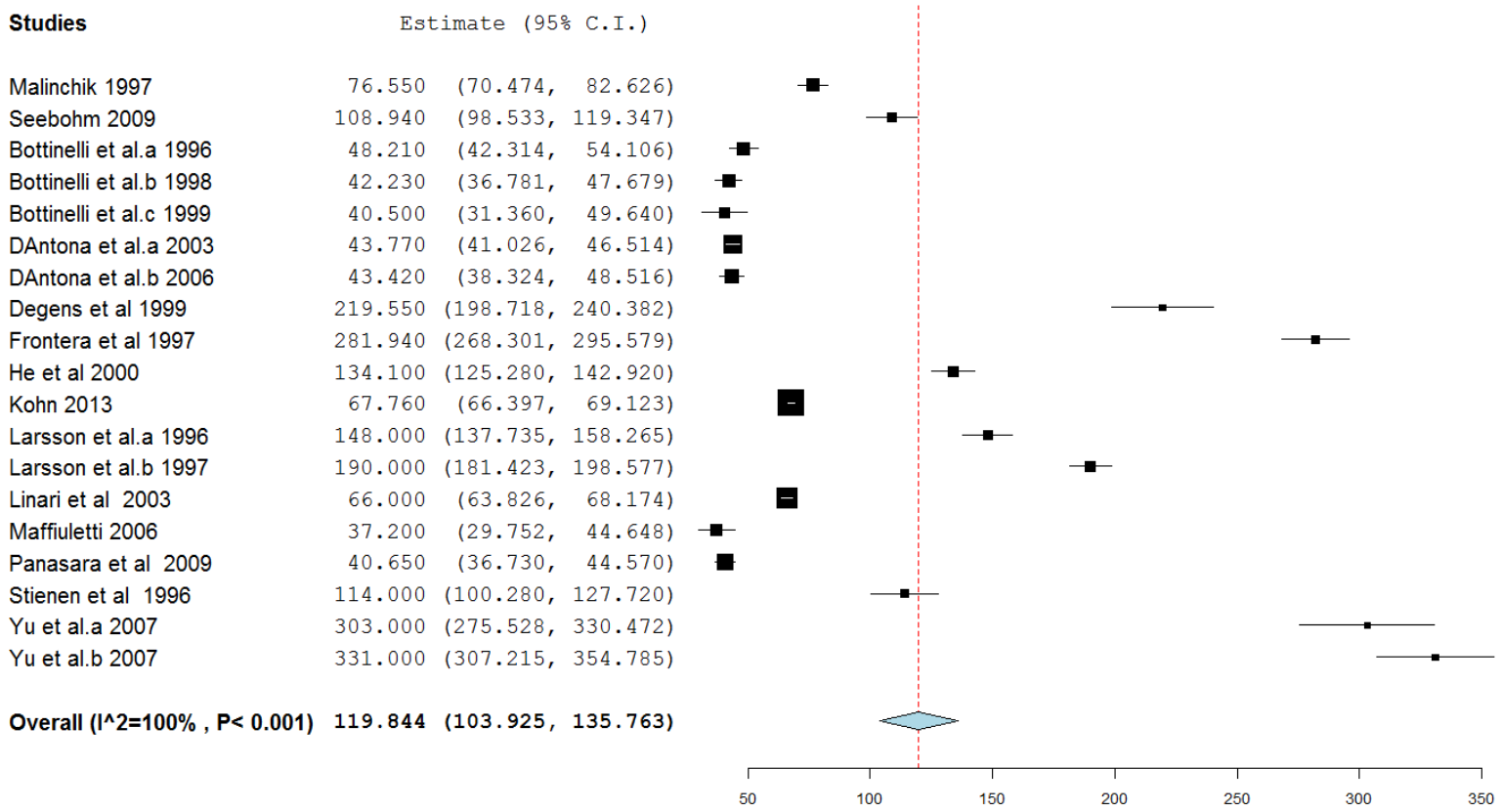


Figure 8.1. A Forest Plot of mean published MHC I fibre specific force results measured at 12°C.

The mean specific force was 120 kPa, and up to 8-fold variability was observed, the largest range of all the data sets outlined in Chapter 2. The χ^2 was significant ($p < 0.05$) and the I^2 of 100% indicates that the heterogeneity in the data sets was extremely high.

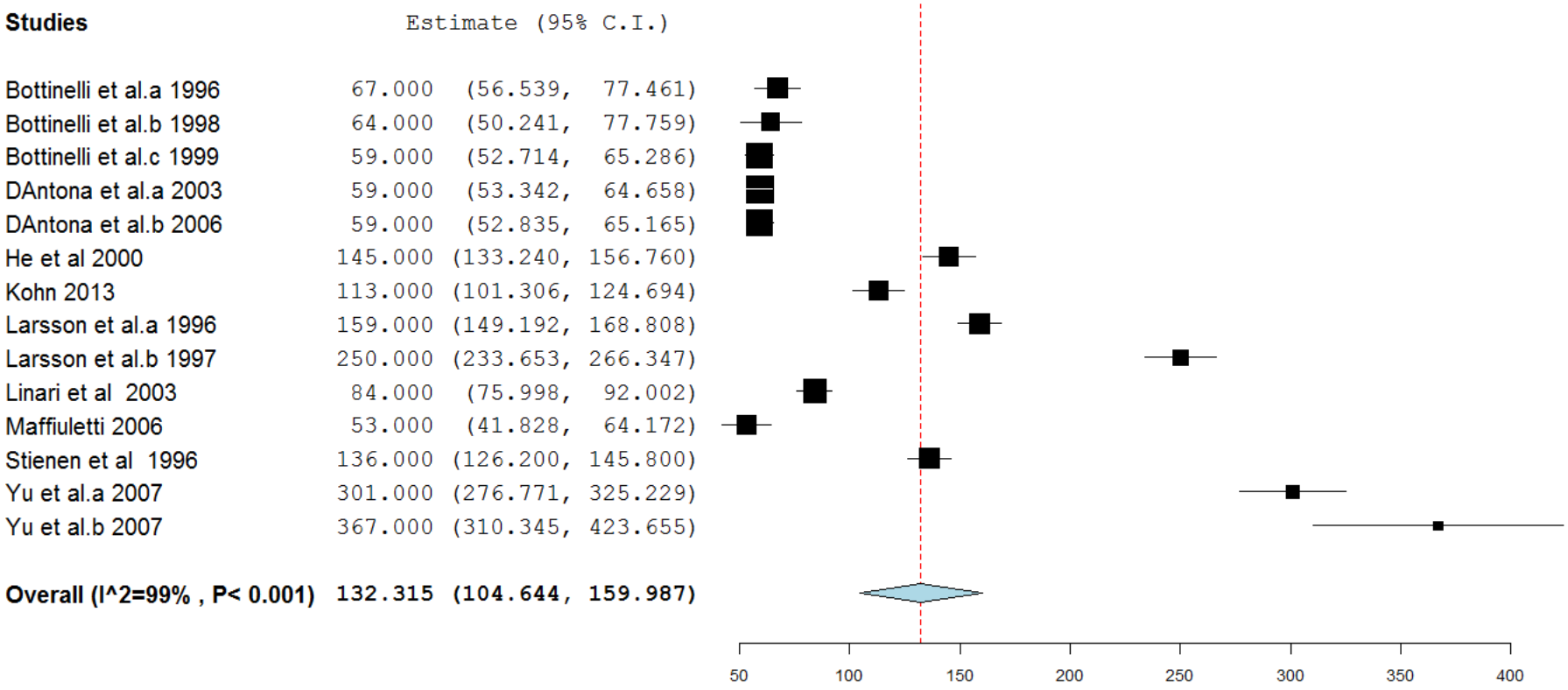


Figure 8.2. A Forest Plot of mean published MHC IIA fibre specific force results measured at 12°C.

The mean specific force was 132 kPa. The χ^2 was significant ($p < 0.05$) and the I^2 of 99% indicates that the heterogeneity in the data sets was extremely high.

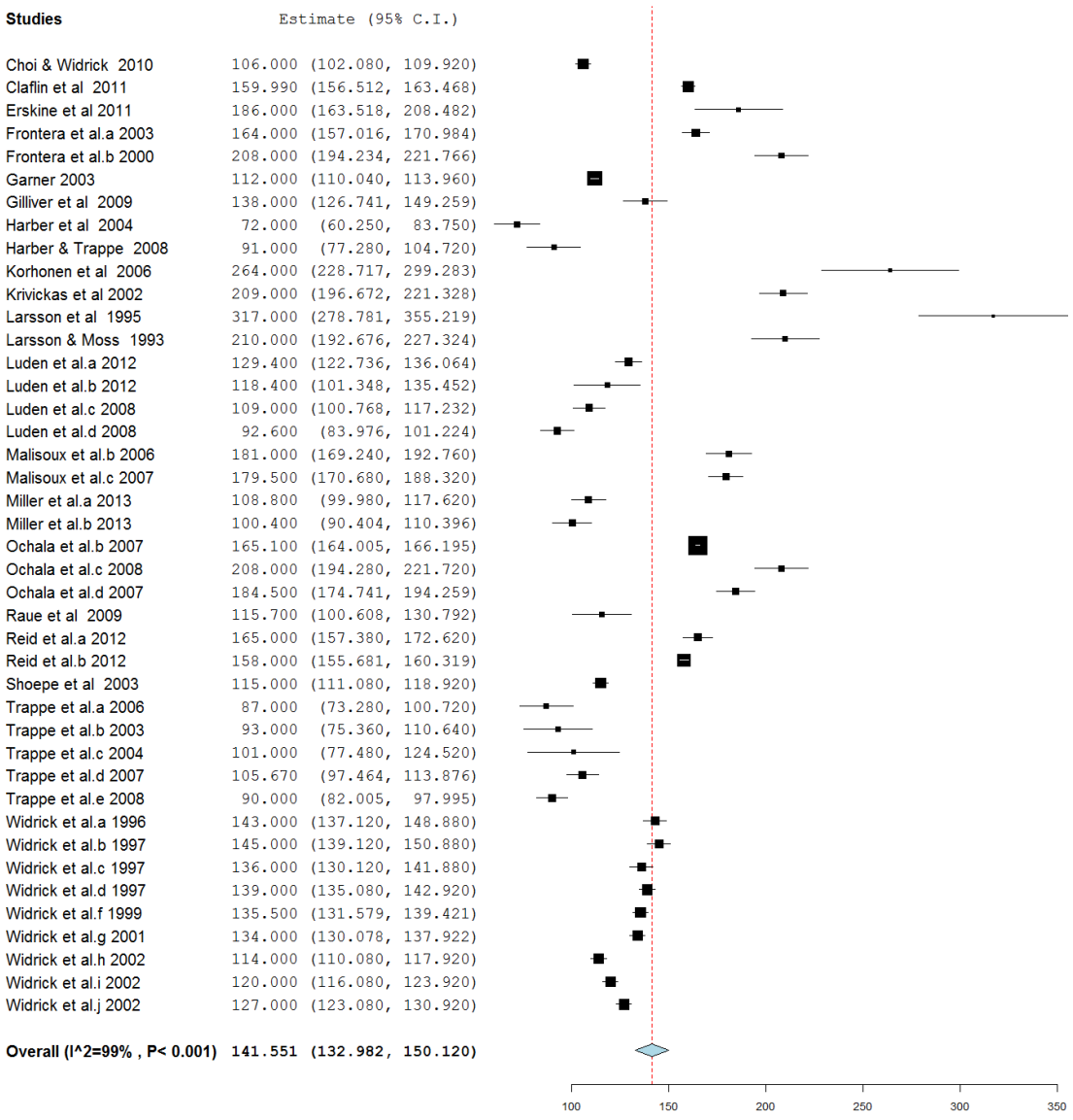


Figure 8.3. A Forest Plot of mean published MHC I fibre specific force results measured at 15°C.

The mean specific force was 142 kPa. The χ^2 was significant ($p < 0.05$) and the I^2 of 99% indicates that the heterogeneity in the data sets was extremely high.

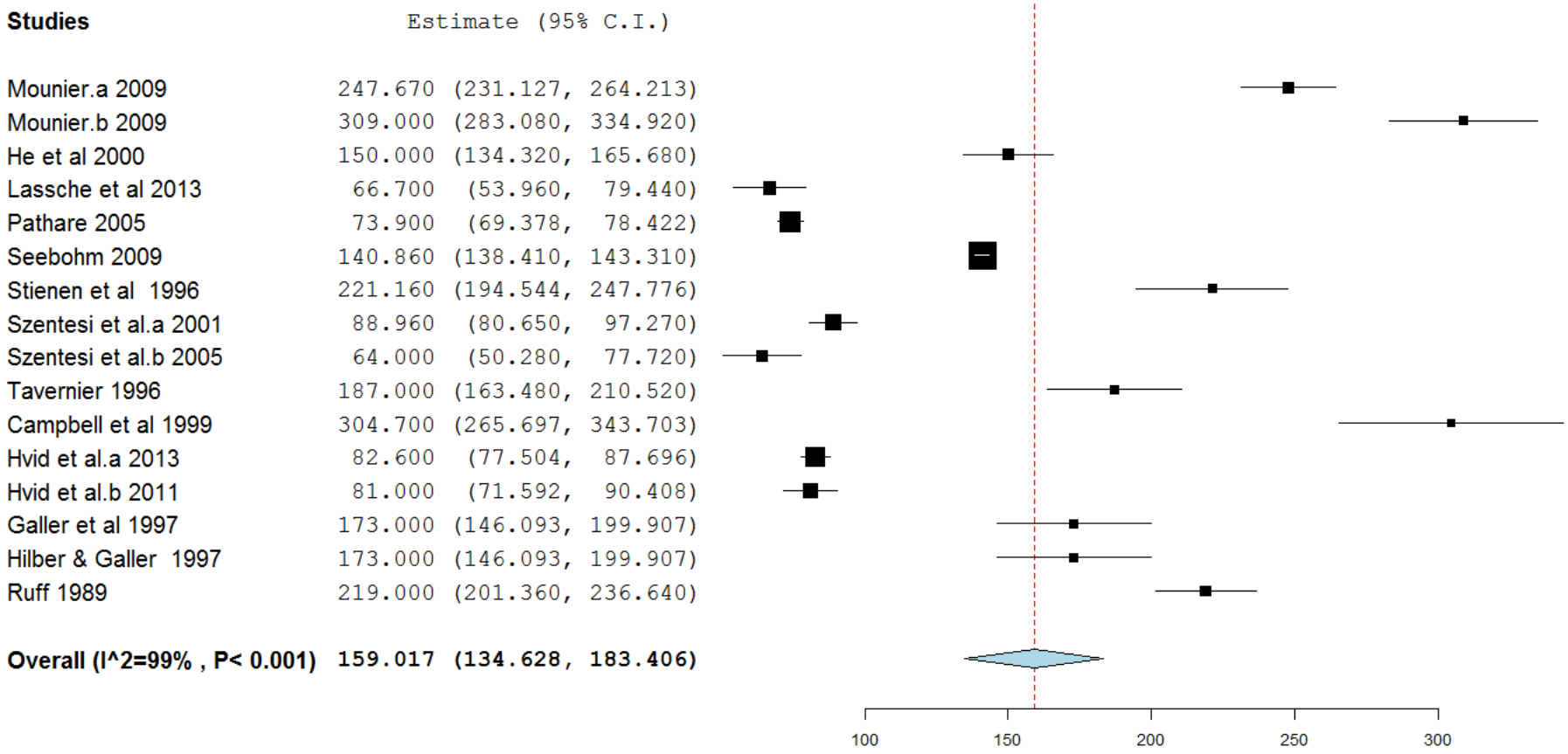


Figure 8.4. A Forest Plot of mean published MHC I fibre specific force results measured at >18°C.

The mean specific force was 159 kPa. The χ^2 was significant ($p < 0.05$) and the I^2 of 99% indicates that the heterogeneity in the data sets was extremely high.

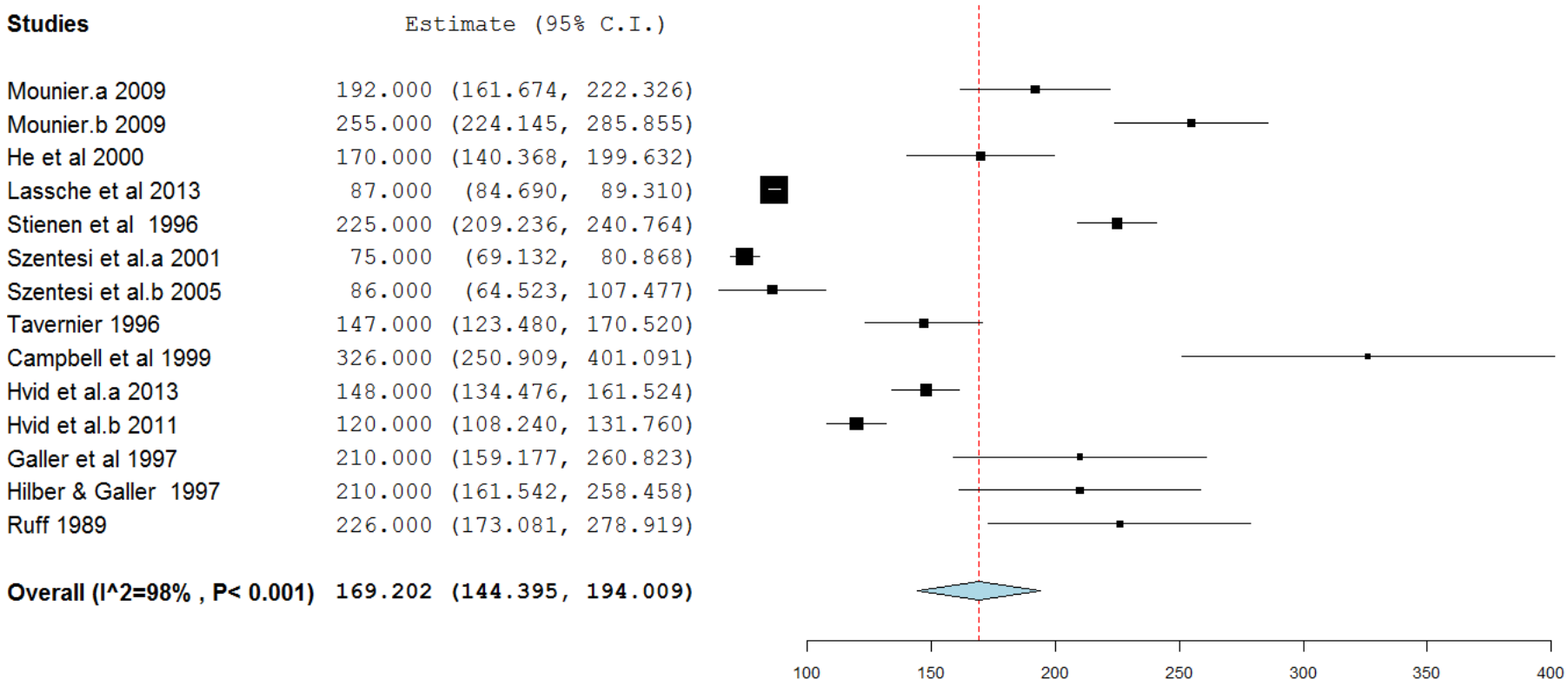


Figure 8.5. A Forest Plot of mean published MHC IIA fibre specific force results measured at >18°C.

The mean specific force was 169 kPa. The χ^2 was significant ($p < 0.05$) and the I^2 of 98% indicates that the heterogeneity in the data sets was extremely high.

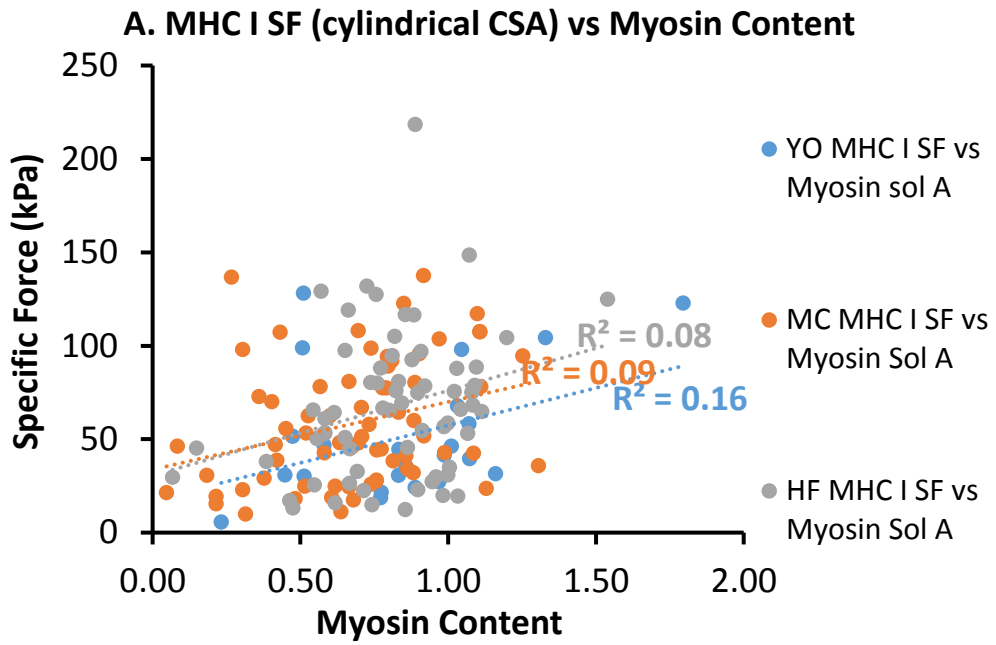
8.3 Correlation between myosin content and skinned fibres from young and elderly experimental groups.

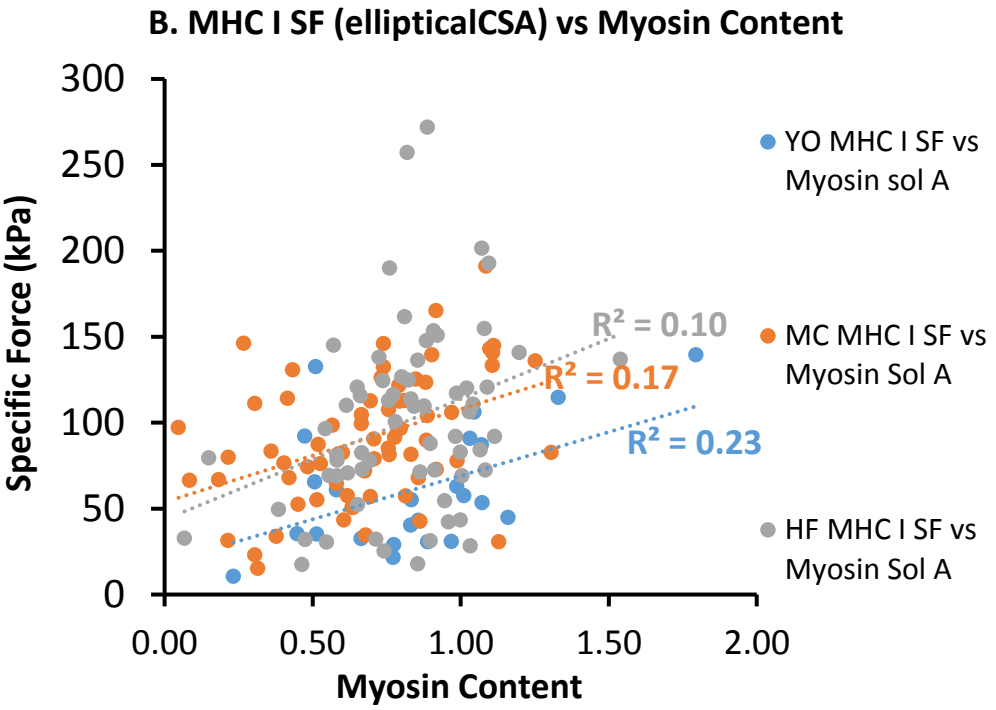
The relationship between the myosin content and specific force measured in both solutions A and B from individual skinned fibres, for participants in each experimental group (Young controls; Master Cyclists; Hip fracture patients), is illustrated in Figure 8.6. Specific force was calculated assuming both a cylindrical and an elliptical skinned fibre CSA for the same skinned fibres, to determine whether the CSA shape assumed affects the correlation between skinned fibre specific force and myosin content.

As can be seen in Figure 1 A and B, the correlation between specific force measured in solution A and myosin content from individual fibres is weak in all groups, when either a cylindrical or elliptical fibre shape is assumed to calculate specific force. In both Figures 1 A and B the correlation is strongest in young individuals compared to both elderly groups. In Figure 1 C and D the correlations between specific force measured in solution B and myosin content were weak in all experimental groups, but strongest in the young group, while no correlation at all was observed in skinned fibres from master cyclists.

The correlations between myosin content and skinned fibre specific force from individual fibres were weak (Figure 8.6), which suggests that little mechanistic information can be gained from this comparison at the skinned fibre level. However, previous studies have demonstrated that myofibrillar protein content (Trappe et al., 2003) and thick filament density (Widrick et al., 1997) differences (or lack thereof) between experimental groups mirror the trends in specific force between the same groups. Specific force was also shown to be proportional to the ratio of myosin:actin in skinned fibres from healthy controls and AQM patients (Ochala and Larsson, 2008). These findings suggest

that more detailed information regarding myofibrillar protein content (i.e. actin as well as myosin) may be required in order to observe stronger correlations between protein content and specific force from individual skinned fibres. The relationship between mean myosin content and specific force of a given experimental group from the present study (Chapter 5) was consistent with that observed by D'Antona et al. (2003). However, a comparison of the correlation between individual skinned fibre specific force and myosin content from the present study and that of D'Antona et al. (2003) could not be made.


A. MHC I SF (cylindrical CSA) vs Myosin Content
Specific Force (kPa)
250
200
150
100
50
0
0.00
0.50
1.00
1.50
2.00
Myosin Content
R² = 0.08
R² = 0.09
R² = 0.16
YO MHC I SF vs Myosin sol A
MC MHC I SF vs Myosin Sol A
HF MHC I SF vs Myosin Sol A


B. MHC I SF (ellipticalCSA) vs Myosin Content
Specific Force (kPa)
300
250
200
150
100
50
0
0.00
0.50
1.00
1.50
2.00
Myosin Content
R² = 0.10
R² = 0.17
R² = 0.23
YO MHC I SF vs Myosin sol A
MC MHC I SF vs Myosin Sol A
HF MHC I SF vs Myosin Sol A

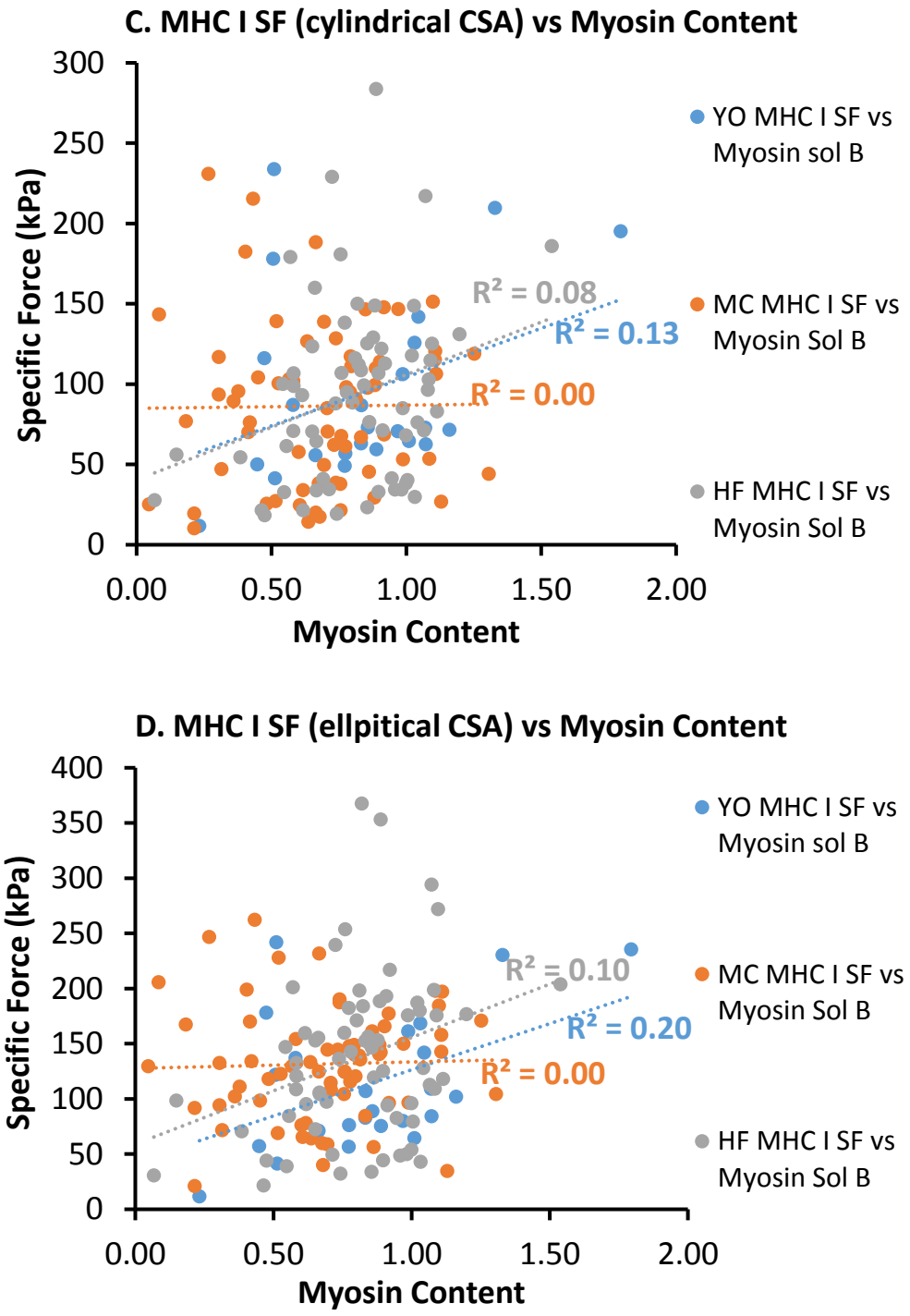


Figure 8.6. Mean specific force from individual MHC I skinned fibres, plotted against the myosin content of the same fibre. Experimental groups are denoted as blue dots (Healthy, young group), orange dots (Elderly master cyclists) and grey dots (hip fracture patients). Specific force values measured in solutions A (A and B) and B (C and D) were calculated assuming both cylindrical (A and C) and elliptical (B and D) cross-sectional shapes for the same skinned fibres. The correlation (R^2) was weak in all cases, but was higher in young compared to elderly groups. When an elliptical cross-sectional shape was assumed (B) the correlation between specific force measured in solution A and myosin content was noticeably higher in master cyclists compared to hip fracture patients, which was not the case when a cylindrical cross-sectional shape was assumed. In solution B, there was no correlation between skinned fibre specific force and myosin content in the master cyclist groups.

8.4 Matlab code used in the present thesis

8.4.1 Script file used to calculate the baseline, P_0 and t_{50} of a given skinned fibre force signal, as used in chapter 4.

```
clear
clc
close all hidden

Dir='enter file name here\';
excelfile=[Dir 'Matlab output.xlsx'];
DirStructure=dir(Dir);
Title={DirStructure.name};
% Go through the files one at a time by using a for loop.
for i= 3:length(Title);
    figure; set(gcf, 'windowstyle', 'docked', 'color', 'w')
    File=Title{i};
    Data=dlmread([Dir File]);
    Time=Data(:,1);
    Force=Data(:,2);

    % smooth the raw data before plotting
    Smooth=smooth(medfilt1(Force, 7),50);
    %plot(Time,Force,'c', Time, Smooth, 'b');
    plot(Time, Smooth, 'b');
    ylim([min(Smooth(10000:end)) max(Smooth(10000:end))])

    %Select start/end of candidate signal
    [T1, Thresh]=ginput(2);

    %Select the minima of the signal as a starting point
    SelectedTimeIdx = find(Time>T1(1),1,'first');
    ROI = Smooth(SelectedTimeIdx:SelectedTimeIdx+1000);
    [~,LocalMinimaIdx] = min(ROI); %Find minima of valley (starting
point)
    Start_Pt = (SelectedTimeIdx + LocalMinimaIdx);
    End_Pt=round(T1(2)*1000);
    hold on
    plot(Time(Start_Pt), Smooth(Start_Pt),'dr')

    Force_trace=Smooth(Start_Pt:End_Pt);
    Time_scale=Time(Start_Pt:End_Pt);
    Time_scale=Time_scale-Time_scale(1);

    % reject the 1st few data points
    min_point = 300; %Remove signal artifact (right after starting
point)
    BL1=min(Force_trace(min_point:end));
    col='rcgy'
    figure; set(gcf, 'windowstyle', 'docked', 'color', 'w')
    % find the point where the slope has a zero y-intercept
    for j=1:100

        if (BL1<0)
            min_point = ceil(min_point*1.005);
            BL1=min(Force_trace(min_point:end));
        end
```

```
        cla
        Force_trace=Force_trace-BL1;
        Peak1=max(Force_trace(min_point:end-30));
        Extrapolation_point=Peak1*0.10;
        D=Force_trace<Extrapolation_point;
        T2=find(D,1,'last');
        [SlpBL2]=polyfit(Time_scale(min_point:T2),
Force_trace(min_point:T2),1);
        Slope=SlpBL2(1);
        BL2=SlpBL2(2);

        plot(Time_scale, Force_trace)
        hold on
        plot([0, Time_scale(T2)], [BL2,
Time_scale(T2)*Slope+BL2],col(1))


        disp(['round number ' int2str(j) ' baseline is '
num2str(BL2)])
        disp(['Min point: ' num2str(min_point)])
       if abs(BL2)<1e-10
          break
       end
       BL1=BL2;
       drawnow
    end
% Plot original signal Overlayed with Starting Point (green) and
baseline (red)
% h_signalOverlay = figure;
% plot(Smooth);
% hold on;
%
figure(h_signalOverlay);plot(Start_Pt:End_Pt,Smooth(Start_Pt:End_Pt),'
g')
%
figure(h_signalOverlay);plot(Start_Pt+min_point:End_Pt,Smooth(Start_Pt
+min_point:End_Pt),'r')
% hold off;

% Calculate final signals
figure;set(gcf, 'windowstyle', 'docked', 'color', 'w')
SMT=smooth(Force_trace, 2000);
TimeScaleFinal = Time_scale(min_point:end);
SignalFinal = SMT(min_point:end);


%Calculate zero point by extrapolating the line from 10% of peak point
P0=max(SMT(300:end-30));
pc10Max = P0 * 0.10;
[pc10MaxIdx] = find(SignalFinal>=pc10Max,1,'first');
[SlpBL2]=polyfit(TimeScaleFinal(1:pc10MaxIdx),
SignalFinal(1:pc10MaxIdx),1);
Slope=SlpBL2(1);
yIntercept=SlpBL2(2);
xIntercept = -yIntercept/Slope;
hold on
grid on

% Adjust signals to zero intercept
SMT = SMT - yIntercept;
```

```
SignalFinal = SignalFinal - yIntercept;

%Plot new signals
x=[-100 100]; plot(x,(Slope.*x),'r--')
plot(Time_scale, SMT, 'r');
plot(TimeScaleFinal,SignalFinal,'b','LineWidth',2)


%Find half width maxima
P0=max(SignalFinal(300:end-30));
halfMax = P0/2;
[halfMaxIdx] = find(SignalFinal>=halfMax,1,'first');
line([TimeScaleFinal(halfMaxIdx) TimeScaleFinal(halfMaxIdx)],[-100
100],'Color','r');

% Maxima of signal
[fullMaxIdx] = find(SignalFinal>=P0,1,'first');
line([-100 100],[SignalFinal(fullMaxIdx)
SignalFinal(fullMaxIdx)],'Color','r');


Time_half_P0 = TimeScaleFinal(halfMaxIdx);
title([Dir File], 'interpreter', 'none')
xlabel([' P0 : ' num2str(P0,4), ' Time to half P0 : '
num2str(Time_half_P0,4)])

%Axis resizing to view full signal
expandAxes = 1.20;
axis(expandAxes .* [min(Time_scale) max(Time_scale) min(SMT)
max(SMT)])

xlswrite(excelfile, [P0, Time_half_P0],'sheet1', ['E' int2str(i+10)])
xlswrite(excelfile, {[Dir File]},'sheet1', ['C' int2str(i+10)])
end
```

8.4.2 Matlab script file used to calculate skinned fibre force, as used in chapter 5.

```
clear
clc
close all hidden

Dir='C:\Users\test\Desktop\Thesis\Ageing study\Investigation into
ageing - skinned fibre force\Young, healthy participants\ASYC-6\ASYC-6
Fibre 15\';
excelfile=[Dir 'Matlab output.xlsx'];
DirStructure=dir(Dir);
Title={DirStructure.name};
% Go through the files one at a time by using a for loop.
for i= 3:length(Title);
    h_fig = figure; set(gcf, 'windowstyle', 'docked', 'color', 'w')
    File=Title{i};
    Data=dlmread([Dir File]);
    Time=Data(:,1);
    Force=Data(:,2);

    % smooth the raw data before plotting
    Smooth=smooth(medfilt1(Force, 7),30);
```

```
    %plot(Time,Force,'c', Time, Smooth, 'b');
    plot(Time, Smooth, 'b');
    ylim([min(Smooth(10000:end)) max(Smooth(10000:end))])

    %Select start/end of candidate signal
    zoom reset; %set default zoom level
    zoom on;
    figure(h_fig); %bring figure in focus
    pause(); % you can zoom with your mouse and when your image is
okay, you press any key
    zoom off; % to escape the zoom mode
    [Tstart]=ginput(2); %select starting region
    zoom out;
    zoom on;
    pause();
    zoom off;
    [Tend] =ginput(2); %select end region
    zoom out;

    %Find the mean of the selected start region
    SelectedTimeStart1Idx = find(Time>Tstart(1),1,'first');
    SelectedTimeStart2Idx = find(Time>Tstart(2),1,'first');
    startSignal = Smooth(SelectedTimeStart1Idx:SelectedTimeStart2Idx);
    meanStart = mean(startSignal);
    %Select the closet point to that mean
    [~,meanStartIdx] = min(abs(startSignal-meanStart));
    %normalises the values around 'zero mean' and get the absolute
values of those (so it doesn't matter if they're positive or negative)
and then find the lowest point, so you get teh closest point around
the mean.
    %meanstartIdx specifies the location of your signal (e.g. it's the
10th
    % element of the signal) and then sets the signal around that
point.

    %Same for end region
    SelectedTimeEnd1Idx = find(Time>Tend(1),1,'first');
    SelectedTimeEnd2Idx = find(Time>Tend(2),1,'first');
    endSignal =  Smooth(SelectedTimeEnd1Idx:SelectedTimeEnd2Idx);
    meanEnd =  mean(endSignal);
    [~,meanEndIdx] = min(abs(endSignal-meanEnd));

    %meanStartIdx =  find(startSignal>meanStart,1,'first');
    %meanEndIdx =  find(endSignal>meanEnd,1,'first');

    Start_Pt = meanStartIdx + (SelectedTimeStart1Idx-1);
    End_Pt = meanEndIdx + (SelectedTimeEnd1Idx-1);

    hold on
    plot(Time(Start_Pt), Smooth(Start_Pt),'dr')
    plot(Time(End_Pt), Smooth(End_Pt),'dm')

    Force_trace=Smooth(Start_Pt:End_Pt);
    Time_scale=Time(Start_Pt:End_Pt);
    % Plot original signal Overlayed with Starting Point (green) and
baseline (red)
    % h_signalOverlay = figure;
    % plot(Smooth);
    % hold on;
```

```
    %
figure(h_signalOverlay);plot(Start_Pt:End_Pt,Smooth(Start_Pt:End_Pt),'
g')
    %
figure(h_signalOverlay);plot(Start_Pt+min_point:End_Pt,Smooth(Start_Pt
+min_point:End_Pt),'r')
    % hold off;

    % Calculate final signals
    figure;set(gcf, 'windowstyle', 'docked', 'color', 'w')
    smoothSpan = 100;
    buffer=ceil(smoothSpan/2)+1;
    SMT=smooth(Force_trace, smoothSpan);
    TimeScaleFinal = Time_scale(buffer:end-buffer);
    SignalFinal = SMT(buffer:end-buffer);
    min_point = 1;
    TimeScaleFinal=TimeScaleFinal-TimeScaleFinal(1);
    SignalFinal = SignalFinal - SignalFinal(1);
    %%% Calculate zero point by extrapolating the line from 10% of
peak point
    %P0=max(SignalFinal(300:end-30));
    %pc10Max = P0 * 0.10;
    %[pc10MaxIdx] = find(SignalFinal>=pc10Max,1,'first');
    %[SlpBL2]=polyfit(TimeScaleFinal(1:pc10MaxIdx),
SignalFinal(1:pc10MaxIdx),1);
    %Slope=SlpBL2(1);
    %yIntercept=SlpBL2(2);
    %xIntercept = -yIntercept/Slope;
    %hold on
    %grid on
    %%% Adjust signals to zero intercept
    %SignalFinal = SignalFinal - yIntercept;
    %%% Plot new signals
    %x=[-100 100]; plot(x,(Slope.*x),'r--')

    plot(TimeScaleFinal,SignalFinal,'b','LineWidth',2)



    %Find half width maxima
    P0=max(SignalFinal);
    halfMax = P0/2;
    [halfMaxIdx] = find(SignalFinal>=halfMax,1,'first');
    line([TimeScaleFinal(halfMaxIdx) TimeScaleFinal(halfMaxIdx)],[-100
100],'Color','r');

    % Maxima of signal
    [fullMaxIdx] = find(SignalFinal>=P0,1,'first');
    line([-100 100],[SignalFinal(fullMaxIdx)
SignalFinal(fullMaxIdx)],'Color','r');



    Time_half_P0 = TimeScaleFinal(halfMaxIdx);
    title([Dir File], 'interpreter', 'none')
    xlabel([' P0 : ' num2str(P0,4), ' Time to half P0 : '
num2str(Time_half_P0,4)])

    %Axis resizing to view full signal
    expandAxes = 1.20;
    axis(expandAxes .* [min(TimeScaleFinal) max(TimeScaleFinal)
min(SignalFinal) max(SignalFinal)])
```

```
    xlswrite(excelfile, [P0, Time_half_P0],'sheet1', ['E'
int2str(i+10)])
    xlswrite(excelfile, {[Dir File]},'sheet1', ['C' int2str(i+10)])
end
```

8.4.3 *Script file used to calculate the chemical composition of solution B and modified activating solutions described in chapters 4 and 5.*

```
clear;
close all hidden;
clc
load SolCalcDat.mat MetsList LigsList NoteList Labels
load LastTab1.mat ForTable1
Tab=  xlsread('SolCalcData.xlsx','Data', 'D9:O19');
DHTab=xlsread('SolCalcData.xlsx','Data', 'D22:O32');
SS=get(0,'ScreenSize');
if SS(4)<950; sc=SS(4)/1020; FS=[10,8]; else sc=1; FS=[12, 10]; end
TabUD=[{Tab} {MetsList} {LigsList} {DHTab} {NoteList}];
B=3; % border width
WFr=1010; HFr=920*sc; % Overall figure width and weight
W1=440; W2=560; % Widths of the 2 columns
H1=380*sc; H2=265*sc; H3=260*sc; H4=100*sc; H5=320*sc; % Hts of the 5
tables (1 and 5 are in the left column)
HL1=(HFr - H1 - H5)/2-B;
HL2=(HFr - H2 - H3 - H4)/3-2*B;
fh=figure('Position',[10 10 WFr HFr],....
          'Color', [0.92 0.92 0.92]);
CN=MetsList';

PPs={....
    [B     H5+4*B             W1        H1]....
    [W1+10 H4+H3+150+6*B      W2        H2]....
    [W1+10 H4+70+5*B          W2        H3]....
    [W1+10 2*B                W2        H4]....
    [B     B                  W1/2      H5]};

APs={....
    [B     H5+H1+2*B            W1     145*sc]....
    [W1+10 H4+H3+150+H2         W2     140*sc]....
    [W1+10 H4+70+H3+3*B         W2      80*sc]....
    [W1+10 H4+2*B               W2      60*sc]....
    [W1/2  B                    W1/2+B  H5].....
    [B     860     W1      55]}; %AP(6) is for the main heading ah6

              %% Heading
ah6=annotation('textbox', 'Units', 'pixels',....
               'Position', APs{6},.....
               'BackgroundColor', [1 1 1],..........
               'VerticalAlignment', 'top',....
               'EdgeColor', [0 0 0], 'LineWidth', 3,......
               'FitBoxToText', 'off',....
               'String', Labels(51:52),....
               'FontSize', FS(2),.....
               'FontWeight', 'Demi');
```

```
            %% user input table (Table 1)

htab1=uitable('Data', BlankCells(ForTable1), .....
              'RowName',[], .....
              'ColumnName',{'Ligand' 'Notes' 'Required|Value'
'in|units' '' 'Stock|Conc mM' 'K+|ratio' 'Na+|ratio' 'Cl-
|ratio'},.....
              'ColumnWidth', {115 35 55 40  2 55 35 35 48}, .....
              'Position',PPs{1} ,.....
              'FontSize',FS(2),.....
              'ColumnEditable', [false,false, true
false,true,true,true],.....
              'CellEditCallback', @FillTables, ..... %  does temp
change
              'CellSelectionCallback', @ShowNote, .....
              'BackgroundColor', [0.9 0.95 1; 1 1 1],.........
              'UserData', TabUD);
rh1=annotation('rectangle', 'Units', 'pixels',....
               'Position',PPs{1},....
               'Color', [0 0 1], 'LineWidth', 3);
ah1=annotation('textbox', 'Units', 'pixels',....
               'Position', APs{1},......
               'FontSize', FS(2),.....
               'BackgroundColor', [0.9 0.95 1],.........
               'VerticalAlignment', 'top',....
               'EdgeColor', [0 0 1], 'LineWidth', 3,......
               'FitBoxToText', 'off',....
               'String', Labels(1:5));

          %% K value Table (Table2)

htab2=uitable('Data', BlankCells2([LigsList num2cell(Tab)]), .....
              'RowName',[], .....
              'ColumnName',[{'Ligand'} ; MetsList], .....
              'FontSize',FS(2),.....
              'ColumnWidth', {115 50 41 41 41 41 41 41 41 41 2 41 2},
.....
              'BackgroundColor', [1 0.95 0.95; 1 1 1],.........
              'Position',PPs{2} , .....
              'CellSelectionCallback', @ShowDetail,.....
              'UserData', TabUD);
rh2=annotation('rectangle', 'Units', 'pixels',.....
               'Position',PPs{2},....
               'Color', [1 0 0], 'LineWidth', 3);
ah2=annotation('textbox',  'Units', 'pixels',....
               'Position', APs{2},......
               'FontSize', FS(2),......
               'BackgroundColor', [1 0.95 0.95],.........
               'VerticalAlignment', 'top',....
               'EdgeColor', [1 0 0], 'LineWidth', 3,......
               'FitBoxToText', 'off',....
               'String', Labels(11:14));


          %% Solution ligand distribution table (Table 3)

htab3=uitable('RowName',[],.....
              'ColumnName',[{'Ligand (L)'}; {'L'}; MetsList], .....
              'FontSize',10,.....FS(2)
```

```
              'ColumnWidth', {100 44 44 38 38 38 44 38 38 38 38 1 38
1}, .....
              'BackgroundColor', [0.9,1,1; 1 1 1],.........
              'Position', PPs{3} );
rh3=annotation('rectangle', 'Units', 'pixels',....
               'Position',PPs{3},.....
               'Color', [0 1 1], 'LineWidth', 3);
ah3=annotation('textbox',  'Units', 'pixels',....
               'Position', APs{3},......
               'FontSize', FS(2),.....
               'BackgroundColor', [0.9,1,1],.........
               'EdgeColor', [0 1 1], 'LineWidth', 3,......
               'FitBoxToText', 'off',....
               'String', Labels(21:24));


           %% Solution other ions table (Table 4)
htab4=uitable('RowName', {'Free (mM)' 'Bound (mM)' 'Total (mM)'},....
               'ColumnName', {'Ca'  'Mg'  'K' 'Na' 'Cl'
'Ionic|Strength'},...
               'FontSize',FS(2),.....
               'ColumnWidth', {60}, .....
               'BackgroundColor', [1,0.94,1; 1 1 1],.........
               'Position',PPs{4} );
rh4=annotation('rectangle', 'Units', 'pixels',....
               'Position',PPs{4},....
               'Color', [0.7 0 0.7], 'LineWidth', 3);

ah4=annotation('textbox',  'Units', 'pixels',....
               'Position', APs{4},......
               'FontSize', FS(2),.....
               'BackgroundColor', [1,0.94,1],.........
               'EdgeColor', [0.7  0 0.7], 'LineWidth', 3,......
               'FitBoxToText', 'off',....
               'String', Labels(31:36));

             %% Recipe table (Table 5)

htab5=uitable('ColumnName', {'Component' 'Volume (ml)'},.....
              'RowName', [],.....
              'ColumnWidth', {120 80 }, .....
              'Position',PPs{5} ,.....
              'FontSize', FS(2),....
              'BackgroundColor', [0.9 1 0.9; 1 1 1],.........
              'ButtonDownFcn', @PrntTabs,....
              'CellSelectionCallback', @Res2TextFile);

ah5=annotation('textbox',  'Units', 'pixels',....
               'Position',APs{5},......
               'FontSize', FS(2),.....
               'BackgroundColor', [0.9 1 0.9],.........
               'VerticalAlignment', 'top',....
               'EdgeColor', [0 1 0], 'LineWidth', 3,......
               'FitBoxToText', 'off',....
               'String', Labels(41:46));
rh5=annotation('rectangle', 'Units', 'pixels',....
               'Position',PPs{5}-[0 0 B 0],.....
               'Color', [0 1 0], 'LineWidth', 3);
          %% Boxes for date and name
htab6=uitable('ColumnName',{},'RowName',{},....
```

```
                'Position',  [B+W1/2 B W1/2-B H5/5],....
                'ColumnEditable', true,....
                'FontSize', FS(2),....
                'ColumnWidth', {200},....

'Data',{'.....................Name...................';date});

FillTables(htab1, [1 1]) % show the default solution
```